INCIDENCE AND PATHOGENESIS OF *ENTEROCOCCUS* ASSOCIATED CHICKEN EMBRYO AND NEONATAL MORTALITY

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ABSTRACT

Chicken embryo mortality and yolk sac infections (YSI) of neonatal chickens cause a significant economic loss to the poultry industry. In recent years, an emergence of *Enterococcus* associated YSI in neonatal broiler chickens was reported in many countries around the world including Canada, but reasons associated with the emergence of enterococcal infections in poultry were not understood. Although *Escherichia coli* was predominantly isolated from dead embryos and YSI in neonatal chickens in the past, enterococci emerged as the main bacterial species from these incidents in the western Canadian poultry industry. Hence, our main objectives were to identify the incidence of enterococci in commercial hatcheries in western Canada associated with embryonic death and to study the pathogenesis of embryonic and neonatal death related with enterococci.

Chicken embryo death could result from many reasons, but as described in Chapter 2, 65.82% of yolk materials from non-viable chicken embryos were positive for at least one bacterial species. Enterococci was predominantly isolated from non-viable chicken embryos followed by *E. coli*. *E. faecalis* was the predominant enterococcal isolate followed by *E. faecium.* While polymicrobial growths were common, 56% of the embryos were co-infected with *E. faecalis* and *E. coli*. We have demonstrated that matrix-assisted laser desorption/ionization time-of-flight mass spectrometry can distinguish enterococci into the species level with 97.18% accuracy, and can be applied for use in routine poultry pathogen identification.

In the third chapter, we have demonstrated that the majority of isolated enterococci and *E. coli* are multidrug resistant and highlighted the urgency of antimicrobial resistance surveillance in this reservoir of non-viable chicken embryos to understand antimicrobial resistance dissemination and its control.

In the fourth chapter, we have utilized an egg infection model to understand the pathogenesis of enterococci and *E. coli*. We were able to demonstrate that *E. faecalis* was able to penetrate the eggshell, and colonize internal organs of chicken embryos without causing inflammation or death. However, once *E. faecalis* co-infected with *E. coli*, it led to high chick mortality due to septicemia and associated increased proinflammatory cytokines prior to hatch. Overall, our research findings highlight the importance of maintaining proper hygienic and biosecurity measures in commercial hatcheries and poultry production facilities in order to minimize bacterial contamination of hatching eggs to control neonatal chicken mortality.

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DEDICATION

I DEDICATE THIS THESIS TO MY BELOVED PARENTS AND TO
MY DEAREST HUSBAND FOR ENDLESS LOVE AND ENCOURAGEMENT

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LIST OF ABBREVIATIONS

AMP	Ampicillin
AMR	Antimicrobial resistant
AMX	Amoxicillin- clavulanic acid
APEC	Avian pathogenic *E. coli*
AS	Aggregation substance
ATCC	American type culture collection
BA	Blood agar
BAC	Bacitracin
BHI	Brain heart infusion
C	Complement
CAM	Chorioallantoic membrane
CARSS	Canadian antimicrobial resistance surveillance system
cDNA	Complementary DNA
CEF	Ceftiofur
CFU	Colony-forming units
CHO	Chloramphenicol
CIP	Ciprofloxacin
CIPARS	Canadian integrated program for antimicrobial resistance surveillance
CLSI	Clinical Laboratory Standards Institute
cpn60	Chaperone 60
CXCR4	C-X-C chemokine receptor type 4
DNA	Deoxyribonucleic acid
ERY	Erythromycin
ESBLs	Extended-spectrum β-lactamases
FLO	Florfenicol
GelE	Gelatinase E
GEN	Gentamycin
GI	Gastrointestinal
H&E	Hematoxylin and eosin

HYL	Hyaluronidase
IL	Interleukin
LB	Luria broth
LIN	Lincomycin
LTA	Lipoteichoic acid
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time of flight mass spectrometry
MDR	Multidrug resistant
MIP	Macrophage inflammatory protein
NaCl	Sodium Chloride
NEO	Neomycin
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells)
PCR	Polymerase chain reaction
PEN	Penicillin
REG3 gamma	Regenerating islet-derived protein 3 gamma
rRNA	ribosomal ribonucleic acid
SEM	Scanning electron microscopy
SPF	Specific pathogen free
SSS	Sulfamethoxazole
SXT	Sulphamethoxazole/trimethoprim
TEM	Transmission electron microscopy
TET	Tetracycline
TLRs	Toll-like receptors
UTI	Urinary tract infections
VAN	Vancomycin
VMR	Vancomycin resistant
VRE	Vancomycin resistant enterococci
YS	Yolk sac
YSM	Yolk sac membrane

Chapter 1 INTRODUCTION AND LITERATURE REVIEW

1.1 *Enterococcus* species

1.1.1 History

Enterococcus was discovered in 1899 by Thiercelin and he described them as Gram-positive, saprophytic diplococci originating from the intestine, and called them as "Enterocoque". He also described the pathogenic potential of this organism in human indicating that translocating of the bacterium via intestines to the bloodstream leads to septicemia. Parallel to Thiercelin's observation, another research group isolated a hemolytic organism associated with endocarditis in a 37 old patient and was named as *Micrococcus zymogenes*, but later this isolate was recognized as *Enterococcus faecalis* and characterized as a hardy bacterium (328). In 1906, MacCallum and Hastings recovered an isolate from a patient with endocarditis and named it *Streptococcus faecalis* to emphasize its intestinal origin. The organism was able to clot milk and ferment sugars including mannitol and lactose but not raffinose. *E. faecalis* was classified under group D streptococci because it had Lancefield group D cell wall antigens (76). *S. faecium* was first described in 1919 and identified as having different sugar fermentation pattern compared to *S. faecalis.* Twenty years later *S. durans* was identified and was characterized by having more restricted fermentation capabilities. The same researchers tried to classify streptococci into four groups named as pylogenic, viridans, lactic and *Enterococcus*. The organisms under enterococci category were able to grow at 10 and 45 ˚C, tolerate high pH in media, grow at high sodium chloride concentration (6.5 % NaCl) and high temperatures as 60 ˚C for 30 min. Under this classification, *S. faecium* was considered as same as *S. faecalis*, but only later in the mid-1960s, it was officially recognized as a separate species. *S. faecium var. casseliflavus* was identified in 1957 as a motile, yellow pigment producing species and followed by the introduction of *S. avium*. In 1984, *Enterococcus* genus was separated from streptococci by using genomic approaches more particularly, deoxyribonucleic acid (DNA)-DNA and DNA- ribosomal ribonucleic acid (rRNA) hybridization studies. The studies demonstrated that *S. faecalis* and *S. faecium* are distantly related to the non-enterococcal streptococci (*S. bovis* and *S. equinus*) of serological group D and to other streptococci; therefore, it was proposed to transfer S. *faecalis* and *S. faecium* to the genus *Enterococcus* (321). The 16S rRNA sequencing confirmed the separation of enterococci from streptococci and lactococci and other Gram-positive bacteria (226).

1.1.2 Diversity

There are over 50 *Enterococcus* species described in the *Enterococcus* genus, these are divided into 5 groups namely *E. faecalis*, *E. faecium*, *E. avium*, *E. gallinarurum*, *E. cecorum* and ungrouped species (119). There were only 17 species identified in 1997, but due to the rise of rapid and novel molecular tools of bacterial identification and differentiation, novel species were included into the *Enterococcus* genus. These new species were isolated from a vast variety of sources. *E. phoeniculicola* were isolated as a novel species from the uropygial gland of the red-billed wood hoopoe (217), *E. ureilyticus* and *E. rotai* were isolated from the environment (323). *E. aquimarinus* was isolated as a novel species from seawater (348). *E.caccae* was isolated from human stools (234) . *E. camelliae* was isolated from fermented tea leaves in Thailand (345). They are ubiquitous in nature where they are present in a vast range of host species including: humans, animals, insects, and plants (82). Hence, it is hypothesized that enterococci descended from the last common ancestor of mammals, reptiles, birds, and insects (140). They also survive in extra enteric habitats including soil, sediments, beach sand, aquatic and terrestrial vegetation, and ambient waters (rivers, streams, and creeks). They may also be considered as heterothermic habitants, in which temperatures are variable, in contrast to the gastrointestinal (GI) tract of warm-blooded animals, where the temperature is relatively constant. They primarily colonize in the GI tract and constitute 1% of the gut microbiota (353). They predominantly reside in the small and large intestines, oral cavity, and stomach (251, 333). They act as commensal organisms potentially helping in digestion and other gut metabolic pathways (52). They are among the commensal population within the gastrointestinal niche. *E. faecalis*, *E. faecium* are abundant in humans while *E. avium* and *E. durans* are found occasionally. The density of enterococci in the colon average 10^7 colony-forming units (CFU) /µg. Enterococci are also present in the genital tract (163). It is noteworthy to mention that enterococci composition may differ with animal species which may relate to their diet (150). They are used as indicator organisms to detect fecal contamination of food and, water since they are shed in human and animal feces (52). Enterococci cause opportunistic infections in humans and animals when the host is immunosuppressed or when intestinal epithelial integrity is damaged (33). Among those, *E. faecalis* and *E. faecium* are predominantly associated with human infections and *E. faecalis* accounts for 80% of these incidences. (173) Other *Enterococcus* species are known to cause human infections include *E. avium*, *E. gallinarum*, *E. casseliflavus*, *E. durans*, *E. raffinosus* and *E. mundtii* (273). They are also

considered as one of the top three nosocomial pathogens worldwide (349). Among these, urinary tract infections (UTI), abdominal and pelvic infections, endocarditis, respiratory tract infections, skin and subcutaneous infections, joint and bone related infections, bacteremia and, meningitis are prominent (336). Neonates, old and debilitated patients are more susceptible to infections. Apart from being a main nosocomial pathogen, enterococci are becoming multidrug resistant (MDR) in the last few decades, and mortality related to antimicrobial resistant (AMR) enterococci infections have increased. *E. faecium* has higher AMR prevalence than *E. faecalis* (173).

Enterococci species have their own host predilection. *E. columbiae* was isolated from intestines of domestic pigeons and *E. asini* from the cecum of donkeys (94, 98). *E. faecalis*, *E. faecium*, *E. hirae*, and *E. durans* are mainly associated with the mammalian gut. An age dependent colonization of *Enterococcus* species in particular animal species is significant. *E. cecorum* is dominant in older birds while *E. faecalis*, *E. faecium*, *E. hirae* are the initial inhabitants (100). The colonization pattern of cattle intestine shows the same pattern as chickens where *E. faecalis*, *E. faecium*, *E. avium* are dominant in calves while *E. cecorum* replace them when they are older (101). A study conducted to determine the enterococci density of pig feces revealed that *E. faecium* and *E. faecalis* are abundant while *E. gallinarum*, *E. hirae*, *E. casseliflavus*, *E. cecorum*, and *E. sulfurens* were in lesser numbers (242). A study conducted on fecal flora of dogs and cats showed that *E. faecalis*, and *E. faecium* were the most prevalent while *E. durans*, *E. gallinarum*, *E. casseliflavus* and *E. avium* were present in low numbers (195). In contrast to this observation, another study noticed that *E. durans* were the predominant isolate from the rectum of cats (175). *E. faecium*, *E. mundtii,* and *E. faecalis* have been reported in feces of the horse (215).

Resident enterococci in wild animals are considered as a reservoir for AMR, more particularly, vancomycin resistance (VMR). A study conducted in feces of wild small mammals in Spain recovered *E. faecalis*, *E. faecium*, *E. gallinarum*, and *E. casseliflavus* where *E. faecalis* and *E. faecium* were the most prevalent enterococcal species. Among the mammals tested, *Rattus rattus* was identified as a potential reservoir for acquired VMR (225). *E. faecium*, *E. casseliflavus* and *E. gallinarum* were isolated from feces of Iberian wolves and Iberian lynx and a low prevalence of VMR was identified (149). Prevalence of enterococci in wild birds have also been studied. Enterococci were detected in 73.8% of the fecal samples analyzed in common buzzards where *E. faecium* was the most prevalent species recognized (48.4%), followed by *E. faecalis* (16.1%), *E. hirae* and *E. durans* (each 12.9%) (294). Enterococci were also isolated from different

fish species and their aquatic environments (104, 243) as well as from different insect species including digestive tracts of cockroaches and flies, where *E. faecalis*, *E. faecium*, *E. hirae*, and *E. casseliflavus* were predominant. It is worth mentioning that except for being a reservoir for enterococci in the GI tract, they transmit resistant traits to the environment (405).

1.1.3 Phenotypic, biochemical and physiological characteristics

Enterococci are Gram-positive non-spore forming cocci that occurs in singles, pairs or chains (120). They are facultative anaerobes and belong to the lactic acid group of bacteria which utilize glucose as the energy source and produce lactic acid. They lack catalase, but they possess superoxide dismutase which detoxifies peroxide radicals. Enterococci are considered as hardy organisms even though they do not produce spores. They can tolerate environmental stresses and have diverse metabolic capabilities which allow them to survive in a wide range of environmental conditions. This inherent property allowed them to colonize various niches and vast range of hosts (297). Enterococci can tolerate a broad range of temperatures (5- 65 ˚C) and pH (4.5-10.0). The ability of enterococci to grow under high salt concentration (6.5% NaCl) is a characteristic feature in this genus (128). The initial studies conducted to differentiate closely related streptococci from enterococci has mentioned that the following features were used as key points to distinguish them; hemolytic ability and tolerance to heat (60 ˚C for 30 min), methylene blue (0.1%), sodium chloride (6.5%), bile, and alkaline medium (pH 9.6). Besides, the action upon milk, esculin, sodium hippurate, gelatin, starch, and many other carbohydrates mainly lactose, inulin, raffinose, mannitol, sorbitol, and trehalose are used in the differentiation of these organisms (24). The ability of enterococci to cation homeostasis probably contributed to their resistance to pH, salt, metals, and desiccation (128).

Enterococci exhibits substrate specific genes which enable them to colonize in particular niches. As an example, *E. faecalis* strain V583 uses mobile genetic elements to utilize sugars and *E. faecalis* strain OG1RF catabolize inositol as a carbon source (297).

1.1.4 *Enterococcus* genome

The first complete enterococcal genome analysis was done in *E. faecalis* V583 strain which was the first vancomycin resistant strain isolated from the United States (241). The mobile genetic elements accounted for more than 25%, of the genome including prophages, integrated and extra chromosomal plasmids, insertion sequence elements, genomic islands including pathogenicity

islands and transposons (285). By observing the genomic structure, it is noteworthy to mention that enterococci can rapidly acquire and disseminate mobile DNA in which most of the elements encode multidrug resistance. The genome analysis reveals the presence of some sugar uptake systems and the genome encodes for a variety of energy production pathways via glycolysis and the pentose phosphate pathway. This enables enterococci to survive in the GI tract. The mechanisms for withstanding oxidative stress and cation homeostasis are explored in the genome analysis. Concerning virulence traits, different surface antigens aiding in colonization and internalization of host cells were identified along with surface structures which aid in immune evasion (285). The *Enterococcus* genome is 3.20 Mb on average, and the average GC content is 37.99%. The core-genome contains 605 genes, the majority of which are associated with carbohydrate metabolism, protein metabolism, DNA and RNA metabolism. The comparative genome analysis of different *Enterococcus* strains from a variety of sources reveals that the habitat is critical in the evolution of *Enterococcus* species where the genetic relationships are closer in strains that come from similar habitats. It was also noticed according to the topology of the time tree, that humans and mammals may be the original hosts of enterococci, and then species from humans and mammals made a host-shift to plants, birds, food and other environments (403). Comparative genome analysis allowed us to understand the difference between commensal and pathogenic strains of enterococci with regards to the genomic diversity. The complete genome analysis of commensal *E. faecalis* isolated from a healthy human infant showed that adaptation to a commensal existence was characterized by lactose and other carbohydrate metabolism genes within genomic islands, accompanied by absence of some major virulence genes including cytolysin but retention of genes for expression of gelatinase E (*gelE*), serine protease, and enterococcal surface protein. The genome analysis of this particular commensal revealed the complexity involved in the explanation of the genomic differences that distinguish commensal and pathogenic *E. faecalis* isolates (47).

1.1.5 Identification methods

Identification and characterization of enterococci species is based on phenotypic, genotypic and phylogenetic techniques (111). The classical phenotypic tests are Gram stain characteristics, sugar fermentation, the presence of specific enzymes, ability to grow in 6.5% NaCl, ability to grow in 10 ˚C and 45˚C, bile tolerance, motility and the presence of Lancefield group D antigens. The colony morphology on specific media including bile esculin agar, esculin iron agar, K-tellurite

agar, and m-Enterococcus agar, can be used to isolate and identify *Enterococcus* species in general. The hemolytic patterns of enterococci can be determined by using media that supplemented with 5% defibrinated sheep blood, horse or human blood agar (BA). The yellow pigmentation produced by some enterococci on trypticase or tryptone soy agar is also used for differentiation. The determination of enterocin production by certain enterococci species is another useful tool. Even though there are commercial kits available to identify enterococci based on the their biochemical characteristics, their routine application is difficult and complex (232). Molecular charactirization of enterocci using polymerase chain reaction (PCR) and sequencing provides more reliable and accurate identification of these organisms into genus and species level. Multiplex PCR have been developed to identifity enterocci species more rapidly and can be used as an alternative to phenotypic identication (18). Gene sequencing of 16S rRNA is considered as the gold standard for enterocci identifcation (202). There are several gene targets including 16S rDNA, RNA polymerase subunit beta (*rpoB*), superoxide dismutase (*sodA*) used as better discriminative tools to identify enrococci into species level (18, 292).

1.1.6 Diseases in humans

1.1.6.1 Nosocomial infections

Enterococci are opportunistic pathogens, and prolonged hospitalization leads to colonization of multiple sites in patients. Alterations in the regulation of the gut immune system by changes the resident microbiota due to continued antibiotic therapy favor settlement of multidrug-resistant enterococci. Enterococcal genomes are incredibly pliable, with the ability to exchange large fragments of chromosomal DNA (318). In addition, the lack of clustered regularly interspaced short palindromic repeats elements plays a potential role in the adaptation of hospital-associated enterococci (260). Enterococci are a leading cause of hospital-acquired bloodstream infections in North America including the USA (56.5%), Canada (55.7%) and Latin America (42.9%) (289). Among those, vancomycin resistant enterococci (VRE) pose a greater threat and cause a significant economic burden worldwide (293, 331). According to the prevalence data available worldwide, there is an increasing trend of enterococcal nosocomial infections and mortality especially due to colonization with MDR isolates (387). Even though *E. faecalis* is the most common encounter in these infections due to its higher pathogenicity, *E. faecium* has accounted for higher mortality due to the prevalence of MDR (173, 267, 290). While traditionally

90% of all enterococcal infections were caused by *E. faecalis* and only 10% were caused by *E. faecium*, the proportion of *E. faecium* has gradually increased over the years to 40% (388). The most common enterococcal infections are UTI, infective endocarditis, abdominal and pelvic infection, bacteremia and surgical wound infections (173, 260, 332). The severity and the mortality rates depend on age, other concurrent infections, antibiotic intakes, immune deficiency and *in-situ* device application (113, 190). Among these infections, enterococci account for 30% of hospital acquired infective endocarditis and pose a significant clinical challenge for the treatment due to lack of effective bactericidal antibiotics. Hence, there is no apparent reduction in the mortality rate due to these infections (267). Elderly patients with degenerative heart valve disease, prosthetic heart valves and a higher incidence of enterococcal bloodstream infections originating from the gastrointestinal or urogenital tracts, have predisposing factors for infective endocarditis (87). Multiple virulence factors involved in translocation, adherence, tissue destruction, host immune evasion and carrying multiple antibiotic resistant traits play a role in the disease development and poor prognosis (240).

1.1.6.2 Multidrug resistant enterococci

Enterococci have the ability to inherently acquire resistance to virtually all antimicrobials currently used. The dissemination of resistant determinants among enterococci and other bacterial species leads to a higher prevalence of MDR strains. Among those, increased incidence of MDR *E. faecium*, in particular, VMR *E. faecium* is higher compared to *E. faecalis* leading to severe infections and subsequent therapeutic failures. The continuous evolution of VRE in their genomic levels is leading to acquire MDR phenotypes. At the same time various environmental and livestock sources act as reservoirs for the most common *van* genes (7). Enterococci are not only resistant to traditional antibiotics including ampicillin (AMP), vancomycin (VAN), high-level aminoglycosides, but also newer antibiotics such as linezolid, daptomycin, and tigecycline (21). The ability of enterococci to colonize the GI tract of hospitalized humans for long periods is a crucial factor that influences the development of drug resistance. Inside the GI tract, enterococci serve as a reservoir for cycles of transmission and spread of antibiotic resistance determinants (284). Enterococci inhabiting livestock reservoirs appear to play a critical role in the acquisition and dissemination of AMR determinants (88) . Enterococci have a wide array of resistant mechanisms which promotes survival in human and hospital environments, including modification

of drug targets, inactivation of therapeutic agents, overexpression of efflux pumps and a sophisticated cell envelope adaptive response (244). Enterococci adaptation mechanisms vary for acquiring resistant determinates mainly via conjugative plasmid and transposons. Since enterococci have the ability to withstand harsh environmental conditions, enterococci facilitates survival and spreading of resistance in the environment (7). Intestinal paneth cells produce regenerating islet-derived protein 3 gamma (REG3 gamma), a secreted type C- type lectin that is produced once stimulated via toll like receptors (TLRs). Gram-positive bacterial cell wall peptidoglycans act as a pathogen associated molecular pattern for TLR stimulation. The production of REG3 gamma leads to the killing of Gram-positive bacteria (including VRE) thus work as a part of the mucosal innate immune defense mechanism. But the application of broad spectrum antibiotics lead to down regulation of REG3 gamma leading to colonization of antibiotic resistant bacteria like VRE resulting in infections (46).

1.1.7 Enterococcal infections in poultry

1.1.7.1 As a commensal in gut

The interactions between the poultry host and their GI microbiota are diverse, and include nutrient exchange, modulation of gut morphology, physiology, immunity, and interaction between individual gut microbes (281). The intestinal microbiome of poultry differs from mammalians as they have a shorter gut and faster food transit time. Immediately after hatch, the poultry GI tract is colonized with exogenous bacteria. The outcome is a complex microbiome which predominantly carries anaerobic bacterial species. As animals age, the complexity and the dynamic nature of the GI microbiota differs and eventually, a stable population is established (48). The poultry GI tract, as characterized by 16S rRNA gene sequencing and next-generation sequencing technologies, identified the predominant bacterial genera to be *Clostridium* species, *Ruminococcus* species, *Lactobacillus* species, and *Bacteroides* species (281). The chicken small intestine is mainly colonized with *Lactobacillus*, *Enterococcus* and *Clostridium* species along with bacteria from the family Enterobacteriaceae. The microbial community of a healthy chicken cecum is more diverse compared to other compartments of the GI tract and is dominated by *Bacteroides* species, *Bifidobacterium* species, *Clostridium* species, *Enterococcus* species, *Escherichia* species, *Fusobacterium* species, *Lactobacillus* species, *Streptococcus* species and *Campylobacter* species (314). Enterococcal population structure and density in the GI microbiota vary significantly with

the age of chicken. Among the enterococcal and streptococcal population in the GI tract of day old chicks, *E. faecalis* and *E. faecium* are the initial colonizers. *E. hirae* and *E. durans* can also be found in the small intestine. While the *E. faecalis* population is rare in 3 to 5 week-old broilers, *E. cecorum* is dominant along with *S. alactolyticus* when they become mature. Even though *E. avium* and *E. gallinarum* were originally described from chickens, they are rarely found. Hence, they are not considered as part of normal gut flora (100). *E. avium* was originally described from human feces (154) but is common in chicken feces (271). Modern poultry production practices, along with extensive use of antimicrobials, influence the enterococci community in the normal flora of chickens (102). In general, *E. faecalis* has a diverse population in the GI microbiome. A study conducted by Olsen et al., 2012 revealed 21 sequence types (STs) in *E. faecalis* from newly hatched chicks. Among those, more than 50% of isolates belonged to three STs demonstrating the adaptive nature of *E. faecalis* strains to a specific avian niche. They showed that the *E. faecalis* population is less diverse at the time of hatch where only 15% of chicks were colonized with *E. faecalis* resembling vertical transmission while 70% chicks colonized within 24 hours (127).

1.1.7.2 *Enterococcus cecorum*

E. cecorum inhabits the chicken gut and is dominant in adult broiler chickens between 3–4 weeks of age (100). They have become an emerging pathogen in poultry including broiler chickens, broiler breeders, waterfowl and turkey worldwide in recent past years (109). *E. cecorum* has a predilection for infecting bones, especially free thoracic vertebra. It can also cause femoral head necrosis and spondylitis. There are significant economic losses due to high mortality and increased condemnations at processing plants due to these conditions (186). Starting from 2002, broilers showed infections with *E. cecorum* in Scotland, Netherlands, Belgium, Canada, the USA, Poland, Germany, and Malaysia (186). Outbreaks of infections in broiler breeders were reported in the USA, Canada, Hungary, South Africa, and Iran; in meat turkeys in Canada and meat ducks in Germany. Now, pathogenic *E. cecorum* appears to be regionally endemic worldwide (183). Even though pathogenesis of the disease course is not yet understood, bacteremia and generalized infection appear to be important steps in the pathogenesis. The bacteria may enter the bird via the airborne route (186). Colonization of the gut with pathogenic strains and evading intestinal epithelium is the first step in the disease process. Vertical transmission of bacteria is suspected but is unlikely (302). The disease process has a septic phase followed by a skeletal phase where

femoral head necrosis and osteomyelitis result in lameness and spinal paralysis at the end. Chicken embryo lethality assays indicate bacteremia and generalized infection appear to be important steps in the pathogenesis of *E. cecorum* infection in broilers (186). Embryos infected with pathogenic strains were unable to control infection and consistently showed gross changes typical of sepsis, including hemorrhage and edema. After 48 hrs of infection, changes were not observed in embryos infected with non-pathogenic strains (45). Genetic analyses of *E. cecorum* demonstrate that strains with increased pathogenicity are genetically related and share several putative virulence genes. Pathogenic *E. cecorum* carry increased AMR compared to commensal strains (183).

1.1.7.3 Enterococcus faecalis

E. faecalis is an early inhabitant of the chicken gut where they dominate in day old chicks, but density is lower when they age (100). Research has shown that there is a high chance of rapid spreading of *E. faecalis* to almost all chickens during hatching if there are contaminated eggs present. They colonize the cloacal mucosa of chickens during hatch. Horizontal transmission occurs through the oral route and by cloacal 'drinking' between chicks in the hatcher (127). Lack of proper egg hygiene including the use of floor and dirty eggs, washed eggs and eggs demonstrating almost invisible cracks represent a major risks for transmission of *E. faecalis* (127). Apart from being a commensal organism, *E. faecalis* is associated with variety of infections including septicemia, endocarditis, and pulmonary hypertension syndrome in broilers (65, 334, 352). As a commensal, *E. faecalis* has a symbiotic relationship to the intestinal microbiota; however *gelE* and serine proteases produced by *E. faecalis* can cause inflammation in intestinal epithelium and disrupt the integrity and normal function. This leads to translocation of normal flora, including *E. faecalis* to systemic circulation and causes bacteremia (351). They have been frequently isolated from dead-in-shell embryos, yolk retentions and yolk sac infections (YSI) predominantly as polymicrobial cultures (316). The genetic investigation of *E. faecalis* strains isolated from first week mortality cases indicated the polyclonal nature of the strains and hence the different sources of infection (274). Inoculation of embryonated eggs at 10 days of incubation via the allantoic route demonstrated that virulent strains of *E. faecalis* led to a higher rate of embryo mortality compared to avirulent strains. Regardless of the degree of virulence, *E. faecalis* strains produced detrimental pathological lesions such as cranial and skin hemorrhage, edema, malformations and growth retardation when inoculated in embryos, (41). Pathogenic strains of *E.*

faecalis are strongly associated with amyloid arthropathy, which was first described in brown layers which led to growth retardation and lameness (210). Since then, unilateral or bilateral polyarticular amyloid arthropathy frequently accompanied by systemic amyloidosis involving mainly the liver and spleen has been reported extensively in brown layers and less frequently in broiler breeders (339). *E. faecalis* has been the most frequently demonstrated pathogen in cases of amyloid arthropathy field outbreaks and is considered to have a significant role in pathogenesis (17). Experimental infection studies with *E. faecalis* were able to reproduce the typical lesions observed in the joints. Infections occur only when the arthropathic and amyloidogenic strain is administered intravenously, intra-articularly, or intraperitoneally and possibly by vertical transmission (211). Natural outbreaks associated with *E. faecalis* seem to be clonal or clonally related as demonstrated by pulsed-field gel electrophoresis (339). Infection experiments suggested a higher infection potency for the small colony variants than for ordinary *E. faecalis* (287).

1.1.7.4 *Enterococcus hirae* and *Enterococcus durans*

E. hirae is a part of the enterococcal commensal community in the GI tract of chickens where their density increases by 3-4 weeks of age (100). It has been associated with increased mortalities, uneven flocks, and subsequent downgrading and increased condemnations. The incidence of *E. hirae* infections may be unnoticed because they occur in young chicks and generate low mortality rates. This may lead to unrecognized outbreaks and poor flock performance and may be attributed to poor chick quality (64). They are mainly associated with brain infections in chicks, vegetative endocarditis in young birds (334, 374) and with septicemia in psittacine birds (99). A study conducted to determine the pathogenicity of *E. hirae* where the bacteria were administered either via the intravenous route to immunosuppressed four day old chicks or to the allantoic cavity of chicken embryos, was not able to reproduce the disease (2). The attempts of disease reproduction in budgerigars has also failed (99). Subsequently, another study showed that when $1x10^8$ CFU of *E. hiare* was administered intravenously to 4-day-old chicks, it led to sepsis, and affected birds showed splenomegaly, hepatomegaly, vegetative valvular endocarditis and mural endocarditis (63). It was also associated with osteomyelitis in the proximal femur of a 3-week-old broiler chicken that also suffered from valvular endocarditis and liver necrosis (205). In mammals, *E. hirae* has been implicated as a probable cause of enteritis in suckling rats, horses and pigs (117).

E. durans is also a normal inhabitant in the chicken gut. They were isolated from the bacterial community in the crop of healthy 1 day old chicks in low numbers but dominated by 3-4 weeks (100). They were also isolated from young chickens with bacteremia and encephalomalacia demonstrating multifocal coagulative necrosis in the liver, areas of malacia in the brain stem, and cerebellar white matter. The bacteria were re-isolated from multiple organs including the brain. But the investigations using the re- isolated strain failed to reproduce the field condition in 1 day old chicks (58).

1.1.7.5 *Enterococcus faecium*

E. faecium is a dominant part of the microflora in the chicken GI tract which reside along with *E. faecalis*. They colonize chicks just after hatching and will maintain in large numbers in the population until birds are 3-4 weeks of age. They can be still isolated from intestines in low numbers even when birds are older (100). They are isolated from dead-in-shell embryos and can possibly be a source of hatching egg contamination (110). *E. faecium* was isolated from acute septicemic infections in 1-2 week old white Pekin ducklings. Gross lesions were primarily hepatomegaly and enlarged necrotic spleens. Fibrinous pericarditis, perihepatitis, and airsacculitis were observed in some birds and mortality ranged from 0.5 to 5%. The disease was reproduced in 8-day-old susceptible ducklings by parenteral infection (317).

1.1.7.6 *Enterococcus gallinarum*

E. gallinarum, which are intrinsically resistant to vancomycin, were associated with endocarditis occurring in native heart valves in human patients and bacteremia in immunocompromised patients (89, 300). They have been isolated in low numbers from intestines of 3-4 week old young chickens and considered as a normal flora (100). They have been isolated from variety of sources related to poultry production and broiler chickens (110).

1.1.8 Putative virulence genes and virulence mechanisms of *Enterococcus* species

Enterococcus species were initially considered as non-pathogenic commensals in humans and different animal hosts. But in favourable conditions, they act as opportunistic pathogens (136). They have become an emerging pathogen in hospitalized patients causing nosocomial infections

and also cause community acquired infections (143, 171). *Enterococcus* species are also associated with emerging infections in animals. The hospital environment, where antimicrobials are used heavily, provides a selective advantage for enterococci that are able to acquire and exchange resistance determinants to compete with other nosocomial pathogens and cause infections. Enterococci are inherently resistant to some antimicrobials which enhances their virulence potential in hospital setups (179). Immunocompromised patients and debilitated patients are at a higher risk of infections as the weakened immune system is unable to cope up with commensal barrier and leads to opportunistic infections. Apart from these predisposing factors, the virulence determinants carried in the core genome and some shared and acquired genes are attributed to enterococcal virulence (136). The pathogenicity of enterococcal infections are not fully elucidated. Different vertebrate and invertebrate models were used to understand the virulence factors and pathogenicity of enterococcal infections (136). The embryo lethality assay is one of the useful tools for understanding the genetic basis of *Enterococcus* virulence. It was successfully used to determine the pathogenesis of *E. cecorum* virulence in chicken embryos (45). *E. cecorum* infection is currently one of the most important bacterial diseases of modern broiler chickens and the pathogenesis of this disease is yet to be understood. The phenotypic and genotypic characterization revealed the separate evolution of pathogenic strains of *E. cecorum* compared to commensal strains. There was a significantly higher embryo mortality observed when embryos were challenged with pathogenic strains isolated from field outbreaks of osteomyelitis compared to commensal strains (185). Embryos infected with pathogenic strains showed gross changes typical of sepsis, including hemorrhage and edema (45). The most common invertebrate models used to study enterococcal pathogenesis were the nematode *Caenorhabditis elegans* and insects. The studies conducted with *C. elegans* showed that *E. faecalis* was able to colonize and grow inside the intestinal lumen without invasion. This can lead to persistent infection and animals die eventually with heavy colonisation of *E. faecalis* (137). The dose depended infections of *E. faecalis* is observed from studies with Greater Wax Moth Caterpillar. The lower doses showed better colonisations while high doses of *E. faecium* inoculums led to death of the larvae (393). A better colonization was observed among some hospital-adapted isolates of *E. faecium* (219).

Secreted factors, surface adhesions, aggregation substances and pheromone-associated virulence factors are the main virulence determinates of the enterococcal genome (179). Cytolysin, GelE, serine protease, extracellular superoxide, hyaluronidase (Hyl) are the main secreted factors

(116, 381). These studies found that *E. faecalis* is a causative agent for colorectal cancer. The extracellular superoxide production leads to mammalian cell instability and chromosomal defects (381). *Enterococcus* species are also associated with the development and maintenance of inflammatory bowel disease where they strongly adhere to intestinal epithelium and form biofilms. They display enzymatic mechanisms protecting them against the effects of reactive oxygen species which help them to survive and induce inflammation in the intestines. The genomic analysis indicates that *gelE* and surface aggregating protein (*asa1*) virulence genes are frequently associated with strains involved in inflamed internal mucosa (147).

Esp-Enterococcal surface protein, Ace-An adhesion to collagen of *E. faecalis*, EfaA- *E. faecalis* surface antigen A, EfmA- *E. faecium* surface antigen A, Ebp-Endocarditis, and biofilm – associated pili, EfaAFfs- Endocarditis specific antigen are the main surface adhesins (189, 220, 354). Other virulence factors include enterococcal capsule, lipoteichoic acid (LTA), and common cell wall polysaccharide: Epa-Enterococcal polysaccharide antigen, bacteriocin, megalopasmids (*E. faecium*), Gls24 (*E. faecalis*), Gls20, Gls33 (*E. faecium*), and peroxidases. These factors are predominantly associated with adhesion, colonization, invasion, tissue damage, inducing inflammation and resistance to host defense mechanisms and inhibition of other competitive bacteria (179). The expression and presence of these factors are higher in clinical isolates compared to commensals (174). The contribution of these virulence factors to the pathogenesis of enterococcal infections were studied in many disease models including, endocarditis, UTI, endophthalmitis, peritonitis, and subcutaneously implanted foreign body models in varying degrees. *E. faecalis* appears to have the capacity to translocate across intact intestinal mucosa in models of antibiotic-induced superinfections (179). Tissue adherence is the first step in pathogenesis of infection. As a commensal organism, binding to the mucosal epithelial cells is essential for colonization and maintaining a stable microbial community in the gut. Enterococcal adhesins promote host cell receptor binding at mucosal surfaces and lead to colonization. They adhere and colonize variety of eukaryotic cells including epithelial cells, endothelial cells, leukocytes and extracellular matrix. After colonization, during tissue invasion, in order to persist and produce infection, enterococci have to overcome host defenses including higher redox potential, limited nutrients and phagocytosis. Hence they express genes favouring their growth inside the host environment and allow them to adhere to host cells and extracellular matrix followed by tissue invasion, immunomodulation and tissue damage. (179).

AS, LTA and surface adhesins aid in adherence and colonization of host cells (189, 346). Apart from aiding in adhesion, AS helps enterococci to transfer phagocytosis and survival within macrophages by suppressing respiratory burst (346). Extracellular secretory factors such as cytolysin, GelE, serine protease, Hyl, extracellular superoxide and LTA are involved in host tissue damage (209, 309, 399). The cytolysin was originally considered as a bacteriocin that acted against Gram-positive bacteria as a lytic toxin lethal for a broad range of cell types including Gram-positive bacteria and erythrocytes, retinal cells, polymorphonuclear leukocytes, and intestinal epithelial cells (81). Hyl leads to host tissue destruction via enzymatic cleavage and promoted spreading of bacteria and their toxins through host tissues. Hence they are called spreading factor (196). LTA, which is a main immune stimulatory molecule of enterococci, can stimulate the expression of tumor necrosis factor (TNF) α in macrophages possibly through the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and p38 pathways and lead to inflammation (380). GelE is able to hydrolyze complement factor (C) 3a in complement pathway and thereby inhibit opsonisation and formation of the membrane attack complex resulting in activation of complement cascade triggered by C3 activation. This leads to immune modulation of host and survival within host (283). GelE can also cleave the anaphylatoxin complement factor C5a and this proteolysis leads to decreased neutrophil migration (357). The ability to form biofilms is a main favourable factor in enterococcal pathogenesis where many genes are involved in biofilm formation. Biofilms allow enterococci to survive on abiotic and biotic surfaces withstanding harsh environmental conditions inside the host. Infective endocarditis, catheter associated UTI have clearly demonstrated the role of biofilms in enterococcal pathogenesis (152, 250). Clinical isolates recovered from nosocomial enterococcal infections showed greater ability to produce biofilms than the commensal strains. Biofilm formation is controlled by multiple, partially overlapping regulatory pathways and multiple factors (206, 250). Infection of catheter-implanted mice with *E. faecalis* induced specific expression of interleukin (IL) 1β and macrophage inflammatory protein (MIP) 1α in the bladder. These responses resulted in a favorable niche for the development of persistent *E. faecalis* infections in the murine bladder and kidney (152).

1.1.9 Immunity against enterococcal infection

The immune mechanisms against enterococcal infections are not yet well established (400). However, the host innate immune system likely acts against enterococcal infection as the first line of defense. A mice peritonitis model was able to demonstrate that myeloid differentiation primary response and toll like receptor TLR2 stimulation lead to neutrophil recruitment to the site of infection and thereby cause rapid clearance of *E. faecium* (221, 222). Complement, activated either by classical or alternative pathways, mediates the neutrophil killing of enterococci (20). Protective antibodies are produced against enterococcal cell wall carbohydrates such as LTA, capsular polysaccharides and microbial surface components recognizing adhesive matrix molecules (319). However, a study conducted using a mouse model demonstrated that intestinal containment and systemic clearance of *E. faecalis* are mediated by the innate immune system while the adaptive immune system is not essential for defense against enterococci. They developed a stable intestinal colonization of *E. faecalis* strain and treated them with ceftriaxone in order to reduce the commensal population in the gut. That led to the expansion of *E. faecalis* throughout the gut and disseminated systemically via damaged gut mucosae. There was no increased population of lamina propria mononuclear phagocytic cells after *E. faecalis* translocation suggesting that these immune cells did not actively translocate the bacteria across the intestinal mucosa. Hence, the adaptive immune response was not evident in *E. faecalis* clearance systemically (66).

Enterococci use several inherent strategies to evade the host immune system. The capsular polysaccharide aids in innate immune evasion. An *in-vitro* study conducted in macrophages by Thurlow et al., 2009 showed that *E. faecalis* serotypes C or D are more resistant to complement-mediated opsonophagocytosis than unencapsulated strains. They demonstrated that the capsule limits the detection of opsonic component C3 on *E. faecalis* cell surface; hence prevents opsonisation and thereby reducing phagocytosis. The capsule masks exposure of the surface antigens, LTA, to the host immune system; thereby reducing the production of TNF-α by macrophages, thus preventing induction of inflammation (356). *E. faecalis* extracellular protease, GelE, is involved in innate immune evasion. GelE has the ability to degrade anaphylatoxin C5a, which is a chemoattractant to neutrophils. The degradation results in decreased neutrophil recruitment to the site of infection and increased survival of enterococci (357). The effect of the GelE in pathogenesis of endocarditis was studied by several researchers. Among those, a rabbit model of endocarditis induced by GelE-producing *E. faecalis* strain demonstrated that GelE lead

to decreased phagocytic infiltration, thus increased bacterial numbers in the heart (355). The factors contributing to persistence of an enterococcal infection is studied using a mouse wound excisional model. It showed that a higher dose of *E. faecalis* leads to increased acute bacterial replication and long term persistence. This persistence is attributed to the presence of peptide resistance factors which resist immune clearance and increase enterococci fitness at the site of infection (68). Enterococci can prevent NF-κB signaling in macrophages thus preventing the production of inflammatory cytokines from these cells. This promotes polymicrobial infections, more particularly in the presence of TLR agonists like *E. coli*. This mechanism is studied in a model of catheter-associated UTI caused by *E. faecalis* and *E. coli* co-infections (358).

1.2 Chicken embryo mortality

1.2.1 Chicken embryo structure and development

The embryonated egg has three main parts including eggshell, albumen, and yolk (376). Germ, the fertilized ovum, is established during the first third of the incubation process, and the nutrients are supplied via adjacent yolk and the albumen. At this stage even though the vascular system is starting to appear, oxygen access is limited to simple diffusion aided by primitive hemoglobin. The energy requirement is fulfilled via glycolysis. The chorion and allantois cavity are developed through the invaginations during the germ development (Figure 1-1). Their membranes converge at the shell eventually, and vessels are developed near to them for efficient gas exchange through shell pores. This allows fatty acid metabolism via transported oxygen to the egg and used as the primary source of energy for embryo development. The vascular system is fully developed shortly after the chorioallantois is complete to assure gas exchange. Fatty acid metabolism provides ultimate energy requirement to complete the embryo development (254). The development of the chicken embryo depends on the nutrients deposited on the yolk and the albumen (367). The albumen consists of approximately 88.5 % water and 10.5 % protein, which is not only used for formation of sub-embryonic fluid, but albumen proteins are known to flow into the amniotic cavity, the yolk sac (YS) and finally the digestive tract of the embryo and are used as the primary source of proteins for tissue synthesis (384). The germinal disc is situated in the yolk. By day 2-3 of incubation, vitelline membrane, which supplies nutrition to the embryo, appears and spreads over the yolk. The yolk consists of approximately 50% water, 15% protein, 33% fat, and less than 1% carbohydrates. During incubation, nutrients pass from the yolk content to the embryo through

the yolk sac membrane (YSM) and its surrounding vascular system. YS internalization starts by day 18 of incubation and is fully absorbed into the chick at day 20 of incubation. The pipping process initiates at day 20 of incubation as the chick pierces the inner shell membrane and breathes in the air cell. The eggshell is the outermost cover of the embryo which is made of calcium carbonate embedded in an organic matrix. It provides around 80% of calcium to the chick at their late stage of development (397). Apart from being a protective shield and a source of calcium, it holds the inner and outer shell membranes (282). The organic matter of the eggshell and shell membranes contain proteins as major constituents with small amounts of carbohydrates and lipids. Pores of the eggshell provide the only communicating channels for the exchange of molecules between the developing embryo and the external environment (295).

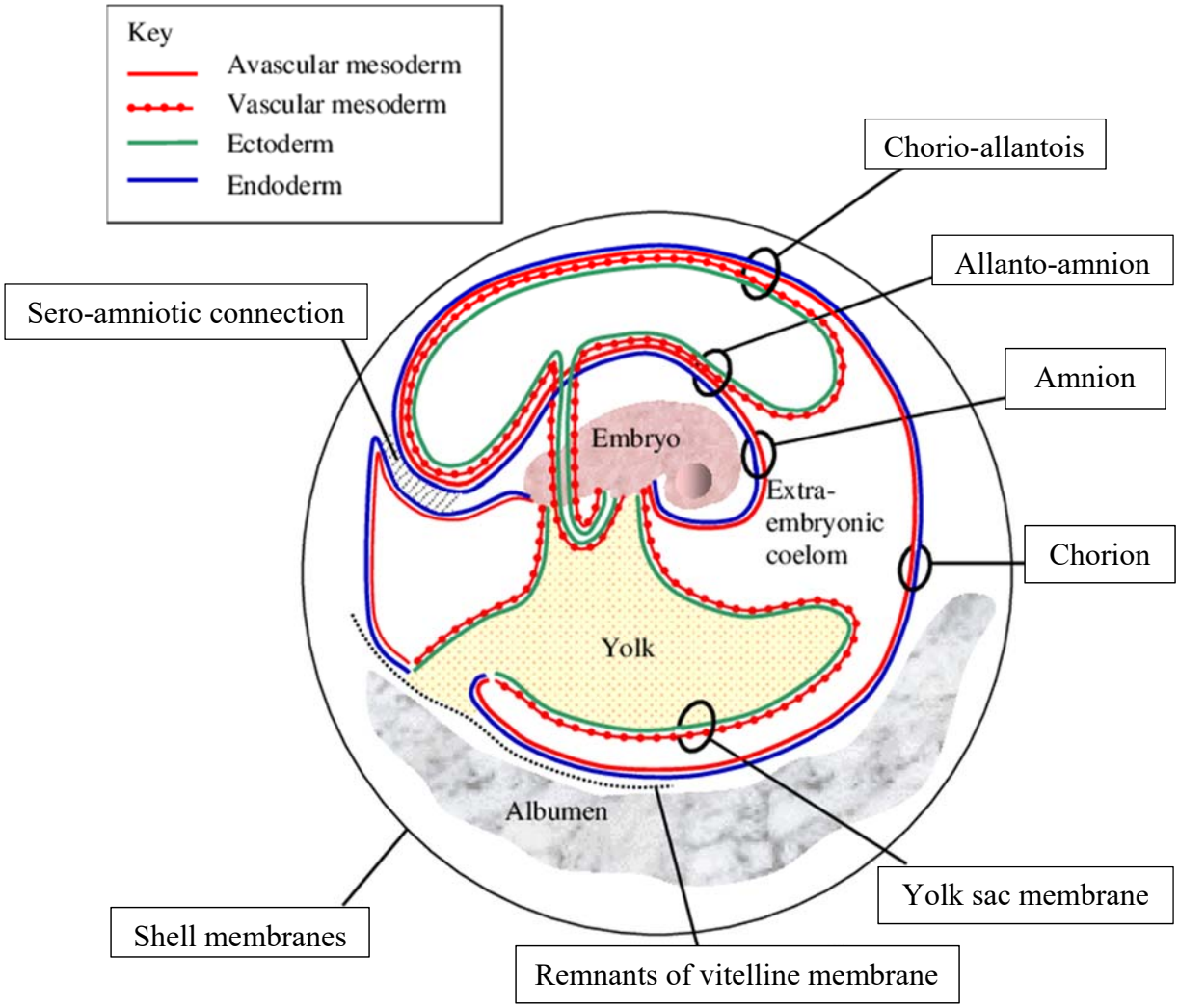

Figure 1.1: A schematic representation of extra embryonic membranes and fluid compartments. Reprinted from Baggott, G. K, Ratite Conference Books. 2001 (28)

1.2.2 Defense mechanisms

The developing chicken embryo is equipped with nonspecific defense mechanisms to protect from microbial invasion (Figure 1-2). These mechanisms are comprised of a complex network of physical and chemical properties of most components of the egg (85). The eggshell, shell membranes and albumen act as primary shields to prevent microbes entering from the outside environment. Eggshell is the outermost covering of the egg which acts as the first physical barrier encountered by microbes. The eggshell is comprised of four morphologically distinct regions including an innermost mammillary zone, followed by the palisade, the vertical crystal layer, and the outermost cuticle. These layers act as physical barriers to bacterial penetration (303). The hydrophobic cuticle which covers eggshell pores acts as the first line of defense (207). The cuticle also provides a chemical barrier against microbial colonization on the eggshell surface and prevents penetration. C-type lysozyme, ovotransferrin, and ovocalyxin-32 present in the cuticle and outer shell, are part of the chemical defense system of the embryo which fights against Gram-positive and Gram-negative bacteria (382). But the bacteria and fungi which have glycolytic activity can digest the eggshell cuticle and facilitate their migration through the eggshell. The cuticle, when it is wet and in its mature state between 6 and 72 hours after an egg is laid, is more effective against bacterial penetration. Cuticles rich in proteins have a decreased shell permeability and greater resistance against bacterial penetration (259). Interactions between inorganic mineral and organic matrix proteins of eggshell establish a unique architecture that prevents most pathogens from accessing the egg interior. This organic matrix contains many antibacterial proteins such as lysozyme, ovotrasferrin, ovocalyxin 21, ovocalyxin 25, ovocalyxin 36, ovocalyxin 32, ovocleidin 17, ovocleidin 116, cystatin3, and avidin (233, 235). They exhibit a strong binding affinity for bacterial polysaccharides (4). These matrix proteins get solubilized during eggshell dissolution along with the embryo development that will provide resistance against bacterial infections at the interface between the eggshell and extra embryonic compartments. The eggshell matrix proteins can disrupt the membrane integrity of several bacteria including *Pseudomonas aureginosa*, *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella enterica* serovar Enteritidis and *E. coli* (245). The proteins involved in chemical defense have broad spectrum bactericidal activities. The egg proteins including ovotransferrin, ovomucoid, lysozyme, ovoinhibitor and avidin which are deposited in the albumen, eggshell matrix, cuticle and the yolk have the ability to chelate vitamins or minerals essential for microbial growth, direct degradation of microbial

components, and inhibit bacterial proteases involved in pathogen invasion (85). The chorioallantoic membrane (CAM) has direct contact with the shell membranes, thus acts as the second physical barrier after the egg shell. CAM is situated just beneath the eggshell lining the structures of the embryo by day 10-13 (270). CAM is likely to express two bacteriostatic proteins, ovotrasferrin and riboflavin binding protein (78). The egg albumen is enriched with antimicrobial peptides namely ovotransferrin, ovomucoid, lysozyme, ovoinhibitor, avidin and cystatin. Ovotransferrin can restrict iron for bacterial growth. Ovomucoid inactivates trypsin enzyme, ovoinhibitor inhibits bacterial and fungal serine proteinase. Lysozyme is an effective bactericidal molecule which destroys the bond between N-acetylmuramic acid and N-acetyl-glucosamine in the cell wall. Cystatin inhibits sulphydryl proteinases while avidin shows a strong binding affinity for biotin. Yolk proteins include lipovitellin, phosvitin, and livetin. Lipoproteins have antibacterial and antiviral properties. Phosvitin has a high metal-chelating ability and livetin (Immunoglobulin Y) binds and immobilize bacteria (4). The antimicrobial peptides avian β-defensins and cathelicidins have a broad spectrum of activity against Gram-positive, Gram-negative, and fungi. The vitelline membrane consists of lysozyme and avian β-defensins which act directly against bacterial species such as *Salmonella*. The total concentrations of ovomucin and lysozyme are 17 times higher in their bacteriostatic and bacteriolytic activity compared to the egg albumen. The amniotic membrane covers the embryo and acts as the last physical barrier. Amniotic fluid contains lysozyme, avian β- defensins 11, and blue mussel shell protein. They demonstrate antibacterial properties which protect the embryo that sits just next to it (164).

Apart from being equipped with non-specific defense components, the innate immune system also plays a role in pathogen recognition and defense against them. TLRs present in embryonic blood vessels, heterophils and macrophages can recognise pathogen associated molecular patterns thus trigger an immune response (230). Progression of innate and adaptive defense systems in a developing chicken embryo is illustrated in Figure 1-3. The first signs of a developing immune system are observed by the day 10 of embryonic age. T cells and B cells are developed on day 11 and 12, respectively. The B cell differentiation occurs after day 15 of age. These events are leading an embryo to become immunocompetent and trigger both innate and adaptive responses against a microbial challenge (320). It is also demonstrated that embryos can innately respond to viral infection through recognition via TLRs which express as early as day 3 of embryonic age. At day 17 of embryonic age, intestinal epithelial cells can respond to

lipopolysaccharides and LTA and enhance the expression of pro-inflammatory genes including IL-6 and IL-18, acute-phase proteins and secretory components from the polymeric immunoglobulin receptor (245). YS serves as a support for the cells of the innate immune system, including monocytes and macrophages, from day 10 and 12, respectively. The generation of phagocytes (e.g., macrophages) in the YS and their infiltration of the embryo have been demonstrated. Macrophages are likely to start residing in liver and kidneys at the age of 12 and 16 days, respectively (248). Embryonic macrophages are actively involved in pathogen recognition and phagocytosis. These cells are associated with blood vessels and at perivascular locations as early as days 4 and 5. They are also present in CAM and respond to microbial infection. The CAM was the first tissue to reveal the presence and action of interferon γ following viral infection and a functioning response to TLR ligands that is present by day 10 -14.

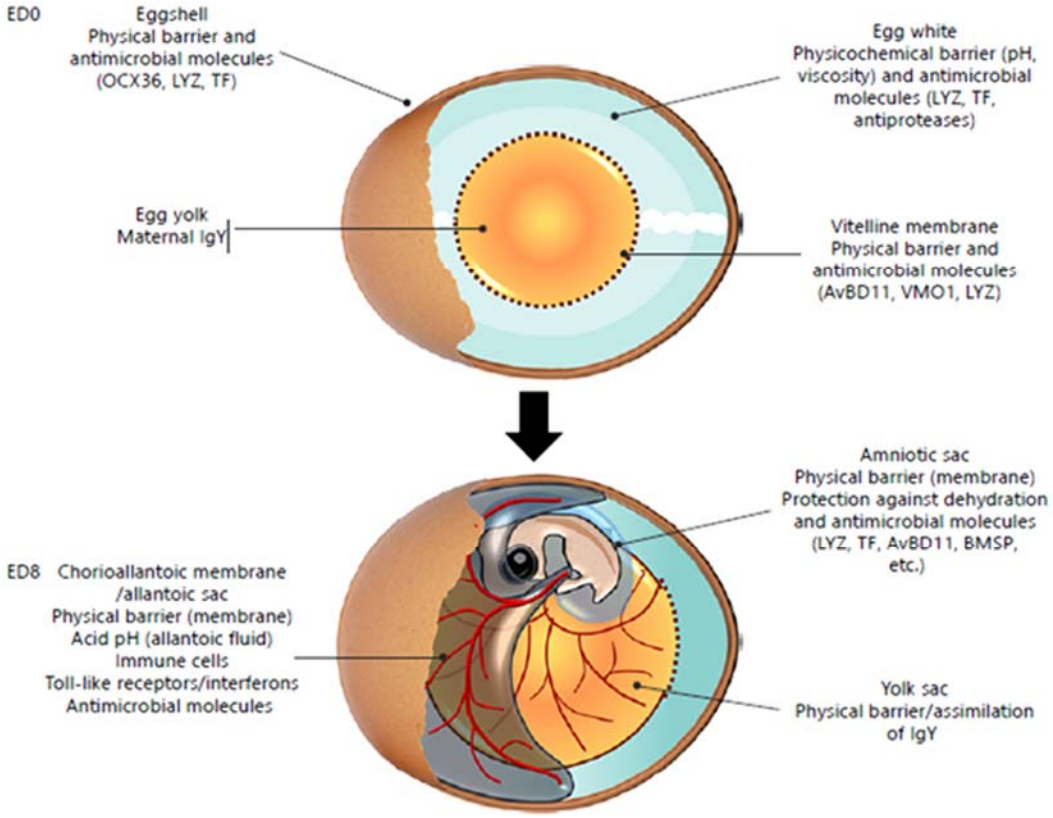

Figure 1.2: The innate defense mechanisms of embryonated egg. Reprinted from Da Silva et al, 2018 (164)

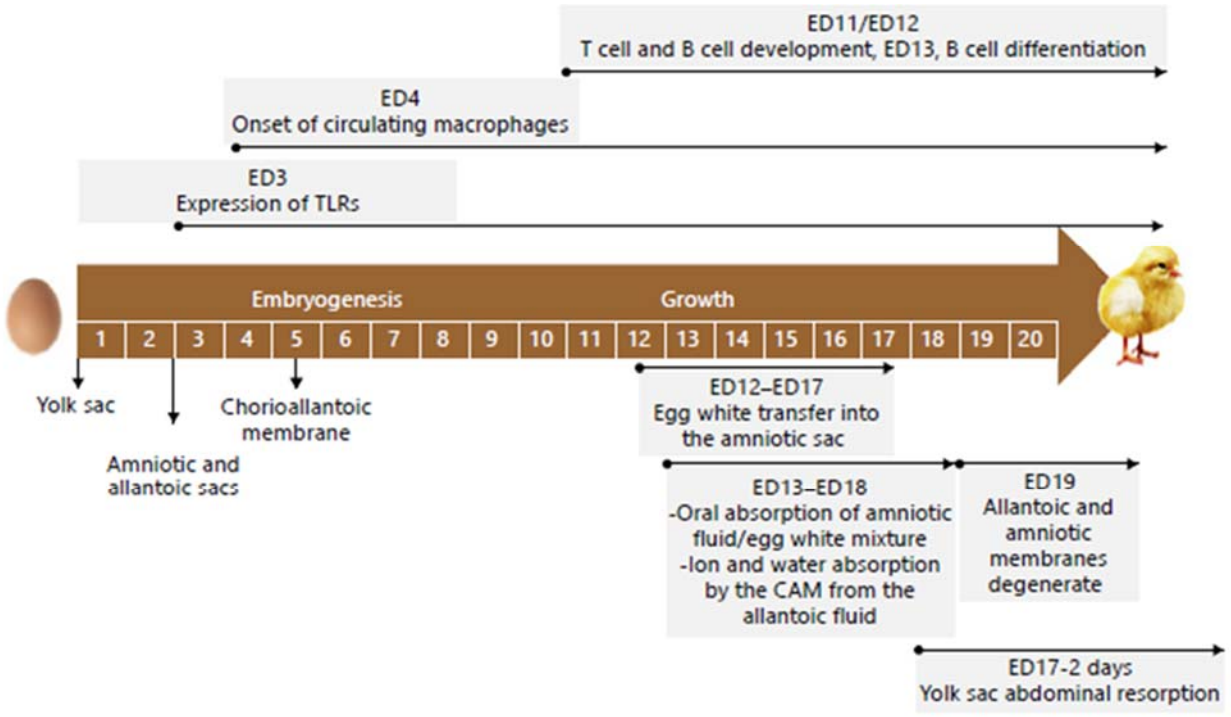

Figure 1.3: Progression of innate and adaptive defense systems in a developing chicken embryo. Reprinted from Da Silva et al, 2018 (164).

1.2.3 Embryo mortality incidence and causes

Chicken embryonic mortality is economically important for the commercial broiler industry. Embryonic death may occur throughout the incubation period and can be a main factor for decreased hatchability (177). In order to improve hatchability rates and provide adequate number of day old chicks to farms, embryo mortality during the incubation period should be reduced. In early years, embryonic mortality rarely exceeds 10% , but rates increased in recent years (307). Three stages of embryonic deaths are described; early (≤ 7 days of incubation), mid (8–14 days of incubation), and late (≥15 days of incubation) (123). Embryonic deaths are not uniformly distributed over the course of incubation, hence two phases of embryonic mortality are described (177). About 65% of embryonic mortality occurs in two phases. The changes in the embryo metabolism along the development process is likely to contribute to these morality patterns. The early phase peaks at day 4 where respiratory maladjustments occur before the chorioallantois is fully developed and functional (301). Carbon dioxide is released from carbohydrate metabolism, which is the main energy source at this stage and tends to accumulate in toxic amounts for the

embryo. Since lactic acid degradation enzymes are low at this early stage of development, its production reaches its maximum level. Nitrogen is released as ammonia in large quantities at this stage and also may become fatal for the embryo. All these events contribute to early phase of embryonic deaths (307). The late phase peaks at day 19 of incubation when the demand for oxygen increases significantly. Events associated with the second phase of embryonic mortality may even cause death for several days after hatching. In the late critical period, failure to transform properly and adapt from allantoic respiration to pulmonary respiration, poor development of the hatching muscle, lack of oxygen in the incubators, excess evaporation of water from the embryo are the leading causes for late stage embryonic deaths. Abnormal changes in the physicochemical state of embryonic fluids predispose for these deaths. Embryonic mortality in chickens is influenced by nutrition, management, hygienic practices and genetic factors (192). Increase in embryonic mortality during incubation is attributed to changes in physiological and developmental functioning of the embryo and has genetic and environmental causes. Eggs laid by old breeders have higher embryo mortality (15). The most common sources of problems include breeder nutrition and management, abnormal egg weight loss during incubation, and bacterial infections (298, 308, 386). Structural abnormalities and malpositions contribute a relatively small percentage of embryonic deaths. Malpositions become evident at or near the end of incubation and are of several types. There are at least four malpositions that make hatching extremely difficult or impossible, either because the chick's head is turned so that the air in the air cell is inaccessible, or because movement is so restricted that the chick is incapable of striking the shell, or because of a combination of both reasons. No chick can hatch if its head is buried between its thighs, a malposition which has been found in 9 to 13% of all embryos dead in the final stages of incubation. It is interesting to note that the incidence of malpositions may be increased considerably by various environmental factors, such as abnormal temperatures and an excess of atmospheric carbon dioxide. These findings indicate that, malpositions are not always the primary cause of death, but may be secondary to unfavorable environmental conditions or other lethal factors. Heredity probably plays but a small part as a cause of malposition (307). Nutritional deficiencies such as amino acids, vitamins and minerals lead to embryonic death. Vitamins D, E, and riboflavin deficiencies lead to death mainly in middle critical period of incubation. Other than non-infectious causes, contamination of incubating eggs with bacteria, virus and fungi leads to embryo mortality and reduced hatchability in chickens and wild birds (157, 275).

1.2.4 Microbial isolation from dead-in-shells

The bacteria which has a pathogenic potential isolated from dead embryos may suggest that they are contributed to YSI, embryonic mortality and reduced hatchability in chickens (17, 275). This association was also observed in wild birds including geese (157). The majority of isolates were Gram-negative bacteria including *E. coli*, *Klebsiella* species, *Pseudomonas* species, *Proteus* species, *Salmonella* species, *Cirobacter* species, *Neisseria* species, *Yersinia* species, *Flavobacterium* species, *Enterobacter* species, *Morganella* species, and *Campylobacter* species The main Gram-positive bacteria isolated from dead-in-shell embryos are *S. aureus*, *Micrococcus* species, *Macrococcus* species, *Streptococcus* species, *Enterococcus* species, *Bacillus* species, and *Corynebacterium* species (17, 157, 275). Among these bacteria, *E. coli* was the predominantly isolated bacteria from dead embryos worldwide. The inoculation of isolated bacteria into the developing embryos to determine the pathogenic potential reveals actual casualty in embryonic death (157). A positive correlation between the presence of virulence genes and embryonic death was observed in *E. coli* inoculated embryos suggesting that expression frequency of these virulence genes is associated with embryo mortality (272). *In-ovo* infection with different routes and doses of *E. faecalis* observed embryonic death at varying degrees (41). There are several mechanisms employed by bacteria to gain entry to the egg. The most likely area on the egg to be penetrated is the air cell end, especially when temperature differential and moisture are favorable (37). The egg defense mechanisms against microbial infections are not always adequate to protect against microbial infections. This will lead to reduce hatchability or embryonic death during incubation (37, 50). Once the bacteria penetrate through the defense barriers of the egg shell, leads to internal contamination. The contamination of egg internal structures occur before oviposition takes place (239). Eggs are contaminated along the reproductive tract and reach yolk and multiply inside the yolk. During oviposition, bacteria in cloaca may be deposited on eggshell and may penetrate inside (338). Once bacteria are internalized, they have to withstand antimicrobial properties of albumen and vitelline membrane before migration to egg yolk. Bacterial growth rate is reduced due to the viscous nature of the egg white proteins, their pH, and the bactericidal properties of lysozyme and conalbumen (239). *S*. Enteritidis is one of the primary contaminants of chicken eggs which has the capability of surviving within the egg (134). When breeders are older, there is a significantly increased level of bacterial contamination on the eggshell surface and in shell pores (257). Trans shell migration of *E. coli* and *Gallibacterium anatis* is studied and

identified that they are highly toxic to the developing chicken embryos (379). Fecal bacteria, including *campylobacter* species, can contaminate the shell, shell membranes, and albumen of freshly laid fertile eggs. This contamination is drawn through the shell by temperature differential and aided by the presence of moisture on eggshell (84).

1.3 Yolk sac infections

1.3.1 Yolk sac structure and development

The YS is a complex extra-embryonic structure that surrounds yolk which is deposited in ovum via receptor-mediated endocytosis of precursor macromolecules synthesized by the liver of the hen (322) . It is primarily responsible for the transfer of nutrients needed for energy and tissue growth from the contents of the YS to the chick embryo. The YS develops from the hindgut of the embryo and begins to envelop the yolk in the initial phase of development (268). Yolk is completely surrounded by the YSM by the 5th day of incubation, and it carries folds equipped with microvilli like structures (337). The YSM is composed of a single layer of endodermal columnar epithelial cells. There are tight junctions between endodermal cells which prevents leakage of intercellular yolk material and seal the extraembryonic compartment from the vitelline circulation. The structure and the number of endodermal cells are varied along the YSM. These structural variations are associated with uptake of extracellular materials. Among those, the mid region is the most active with numerous microvilli, bristle-coated pits, vesicles, apical canaliculi, and vacuoles to efficiently uptake nutrients. The close association between vitelline blood vessels and the endoderm is always maintained throughout the embryo development and small blood vessels developed between the vascular surface of the endoderm and the walls of the large vitelline vessels mediate nutrient uptake and transport. (248). YS starts to develop from ectodermal cells which spread over and fully cover the yolk material. Then, the endodermal cells proliferate in-between the ectodermal cell layer and yolk material and give rise to tight epithelial cell layer in close contact with the yolk. This is followed by migration of cells in splanchnic mesoderm into the spaces between ectoderm and endodermal epithelial cell layer. The growing splanchnic mesoderm forms a network of capillaries in close contact with the ectoderm and endodermal layers. Eventually, these three layers coordinately contribute to the development and transfer of yolk-derived nutrients to the embryonic circulation (31) (398).

1.3.2 Yolk sac functions

The YS is primarily responsible for nutrient uptake from the yolk material and deliver them to the developing chicken embryo (367). The endodermal cells lining the YSM express nutrient transporters and digestive enzymes which facilitate uptake and modify fatty acids, amino acids, peptides and carbohydrates before loading into the circulation. They are actively involved in absorption and transport from embryo day 13- 20 (391). YSM absorb nutrients via endocytosis (Figure 1-4). The yolk droplets containing particles of 25 to 30 nm very low-density lipoprotein particles can be mainly endocytosed via endodermal cells. Then, inside the epithelial cells lipid molecules undergo hydrolysis and re-esterification before secretion in to the circulation. Once endodermal cells uptake and degrade lipoproteins and other macromolecules, they *de novo* synthesize and secrete lipoproteins which are different from the molecules present in the yolk and secrete to the adjacent circulation (31). Yolk lipid uptake is slow during early incubation while the process becomes very rapid by the late stages of development. YSM is considered to act like intestinal epithelial cells as they express genes for carbohydrate, amino acid and mineral absorption from the yolk contents and secretion to the circulation. But, the exact mechanisms of uptake of these molecules are poorly understood. As the energy requirement of the embryo near hatch is dramatically increased, YS synthesizes glucose as an energy source and stores glycogen in the yolk material. This stored glycogen is delivered in high quantities to the embryo for facilitating hatching; even more than the liver supplies (367). The endodermal epithelial cells of the YSM undergo morphological and functional transition along the embryo development period. The expression pattern of the proteins involved in uptake and secretion of lipoprotein and other macromolecules differ along this transition. Among those, the expression patterns of molecules involved in the nutrient transfer, including endocytic receptor complexes, apolipoproteins, and lipid droplet-associated proteins differ between in area vitelline and area vasculosa. The nutrient receptor triad, LRP2-cubilin-amnionless is an example of being significantly changed their expression levels during this transition. This differentiation process ensures embryo nutrition via yolk uptake and lipoprotein secretion by endodermal epithelial cells (31).

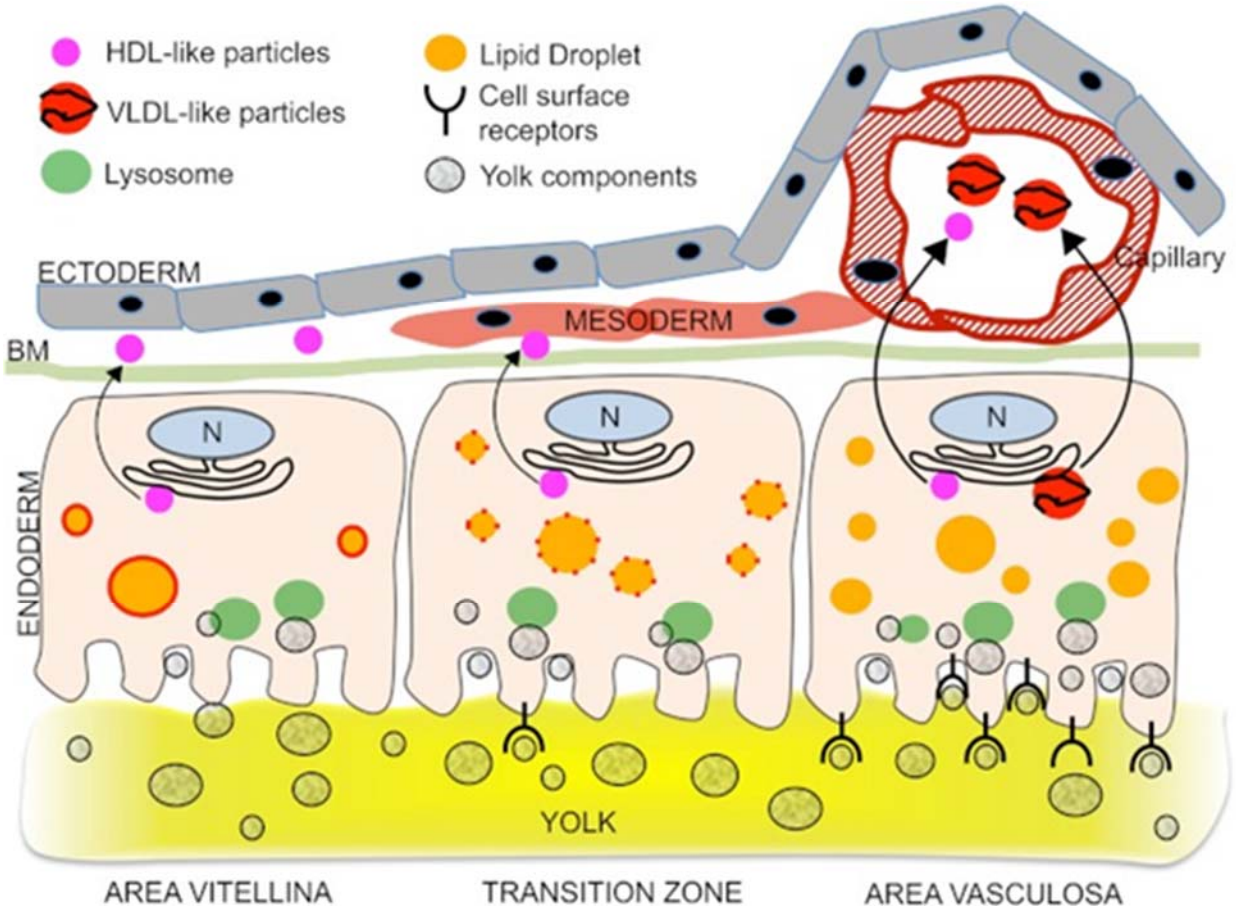

Figure 1.4: The developing chicken YS and nutrient transport across endodermal cells. Reprinted from Bauer et al, 2013 (31).

Apart from being the main nutrient provider to the developing embryo, the YS also functions as a hematopoietic site. As the expansion and differentiation take place, it is the YS which is the major erythropoietic and granulopoietic site in the embryo. While erythrocytic lineage expansion occurs between embryonic days 6-19, granulopoietic expansion occurs between embryonic day 7 and 20 (151). YS also resembles liver functions as they produce carrier proteins and coagulation factors from embryos between days 13- 21. The endodermal epithelial cells also have the ability to catabolize heme and synthesize bile during days 15- 21 of incubation (391).

1.3.3 Yolk sac infections; Incidence and causes

YSI are one of the leading causes of chick mortality accounting for large economic losses to the poultry industry (130). There is a considerable increase in first-week mortality due to YSI

as mortality can go up to 5-10%. Infected birds show local inflammation at the naval and systemic signs leading to septicemia. When the YS becomes infected, they are often described as yellowish-brown to green to yellowish red. The affected birds show distended abdomen and moist umbilicus (200). *E. coli* is the predominant bacteria isolated from infected birds (approximately 70% of cases), however *S. aureus*, *Salmonella* species, *Streptococcus* species, *Enterococcus* species, *Enterobacter* species, *Klebsiella* species, *Pseudomonas* species, *Proteus* species, and *Aspergillus fumigatus* are also frequently isolated (16, 80, 199, 298). It is difficult to establish, which species of bacteria acted as the primary factor and which one acted as a secondary opportunist (200). Extra intestinal *E. coli* strains isolated from YSI were characterized in order to identify the possible virulence determinants associated with adherence and invasiveness which are related to pathogenicity of YSI (204, 310). Intra yolk inoculation of *E. faecalis* to day old chicks resulted in yolk retention without showing signs of inflammation. The yolk proteins get degraded and hydrolyzed by the proteolytic activity of *E. faecalis* in infected YS. This observation leads one to hypothesize that bacterial contamination of the YS is associated with decreased rate of yolk macromolecule and antibody absorption (316). The chicks are most commonly affected during the first week of their lives while mortality peaks at 3- 4 days of age. Bacteria mainly gain access to the YS via unhealed naval or occasionally through haematogenous route (200).

Bacterial contamination can occur during incubation, while hatching or in the brooder. Eggshell contamination during incubation is due to unhygienic conditions in incubators, cross-contamination with hatchery equipment, dirty egg setting, improper handling of incubating eggs during transfer and egg candling. The exploding eggs are one of the main sources of embryonated egg contamination in incubators. Contamination of chicks during hatching occurs due to improper cleaning of the premises or cross-contamination between infected and normal chicks via an airborne route or direct contact. Unabsorbed YS after hatching is a predisposing factor for YSI (200). It is also suggested that isolation of bacteria from YS from chicks during hatching or just after hatching indicates colonization, but not an infection. But more research is warranted to confirm this observation (97). The YSI is experimentally reproduced through different routes of infection including intra yolk, intraperitoneal, naval swabbing, subcutaneous and egg dipping in bacterial broth (340). Birds with subclinical YSI have reduced body weights as the energy is utilized to defend against the infection. When birds cannot control the infection, it leads to septicemia and causes acute form of massive moralities (122).

1.4 *E. coli* infection in chicken embryos and neonatal chickens

Avian pathogenic *E. coli* (APEC) Pathotype, which belongs to extra intestinal *E. coli* is responsible for *E. coli* infections in chicken. The main infection caused by APEC is colibacillosis, an acute, systemic disease which causes significant economic losses to the industry. It is a complex syndrome that includes respiratory tract infection, air sacculitis, pericarditis, perihepatitis, splenomegaly, YSI and swollen head syndrome in broilers (13). In mature laying hens, reproductive tract infection leading to salpingitis or salpingo-peritonitis syndrome is common (77). Environmental factors and contracting viral infections predispose the chickens for colibacillosis (188). The most prevalent serotypes are O1: K1, O2: K1 and O78: K80. However, the number of published *E. coli* serotypes implicated in colibacillosis are increasing. APEC strains are fully equipped with virulence factors involved in adhesion, colonization, translocation, cell damage and resist host immune responses. F1- and P-fimbriae aid in adherence to respiratory epithelial cells. As they need iron to thrive in the host extracellular environment, APEC strains have siderophores which enable sequestration of iron from the host. Aerobactin and yersiniabactin are the main types in iron acquisition system in APEC strains. Hemolysins and temperature-sensitive hemagglutinin cause cellular damage and lead to *E. coli* dissemination in the body. *E. coli* outer membrane proteins and K1 capsule, aid in serum resistance and evasion of phagocytosis. Heat stable toxin, cytotoxin, verotoxin, and flagella toxin are the main toxin products involved in host cell damage. The main port of entry of APEC strains is the air sac epithelium. Once *E. coli* gain access to the respiratory epithelium, they adhere via F1 fimbriae. After colonization, they enter the systemic circulation and cause septicemia (103). *E. coli* is the predominantly isolated bacterial species from dead-in-shell embryos (79). Tran shell migration of *E. coli* either alone or as co-infection with *Gallibactrium anatis* has been demonstrated via egg dipping technique. *E. coli* was able to highly colonize embryos and lead to 100% mortality in 48 hrs post-infection (379). Inoculation of *E. coli* strains isolated from colibacillosis cases to 12 day old embryonated eggs via the allantoic route demonstrated the pathogenicity of *E. coli* in developing embryos. Infected embryos showed encephalomalacia, cranial and skin hemorrhages and a higher mortality rate compared to avirulent strains (389). Embryos inoculated with *E. coli* also demonstrated decreased hatchability, lower body weights, and increased yolk-to-body weight ratios and increased mortality compared to control groups. *E. coli* was re-isolated from the yolk and systemic organs after few days of

infection (253). Even though pathogenesis related to *E. coli* YSI is not yet understood, detection of virulence factors of *E. coli* strains revealed factors associated with invasiveness might be important in YSI pathogenesis (311).

1.5 Poultry industry in Canada

The poultry industry is one of the most popular animal husbandry practices worldwide. The growing consumer demand led to the continuous growth of poultry production since 2014. The global poultry production was 95,594 thousand metric tons in 2018 and the world poultry production is forecast to grow 2% in 2019 to a record of 97.8 million tons. The United States is the highest chicken meat producing country (368). In Canada, chicken is the most consumed animal protein source and the demand for poultry and egg products is growing tremendously. Since 2013 the contribution to the food availability increased by 1.9% for chicken, 0.6% for turkey, and 3.3% for eggs in Canada. The individual chicken consumption was 33.1 kg in 2017. According to the statistics conducted in 2017, there were 2,836 chicken producers and among them, 26.5% were in western Canada. There are 241 Broiler hatching egg producers and 1,059 egg producers in Canada. Among them, 10 hatching egg producers are located in Saskatchewan. There are 35 broiler hatcheries and 20 located in western Canada (53). In 2017, Canada produced poultry and egg products worth $4.4 billion which accounted for 1.37 billion kilograms of commercial chicken and turkey meat production. Poultry industry contributes to the growth of the Canadian economy by being a main exporter worldwide. Among the export market, the United States is the largest market. In 2018, Canada exported 125 thousand metric tons and in 2017 Canada was able to export over 14.7 million chicks and poults, worth $56.0 million to 32 countries. There were over 39.8 million hatching eggs worth over 68.8 million dollars exported to 22 countries (5, 368).

1.6 Antimicrobial resistance of enterococci

Development of resistance to medically important antimicrobials is a global health concern (361). The emergence of MDR bacteria more particularly in hospital settings leads to a higher number of human deaths worldwide. Among them, VRE strains, which are generally MDR, are a leading cause of nosocomial infections and subsequent deaths. Bloodstream infections and other infections including UTI and wounds due to VRE are growing in Canada. According to the Canadian Antimicrobial Resistance Surveillance System (CARSS) report published in 2018, VRE

isolates demonstrated a substantial increase in resistance to daptomycin, high-level gentamicin and nitrofurantoin (55). Apart from colonizing humans, inhabiting animal reservoirs and the environment, VRE are the possible causes of acquisition and dissemination of antibiotic resistance determinants (88). The CARSS indicates that nearly four times greater amount of antimicrobials were used for animal husbandry practices compared to use in humans (55). This overuse or misuse probably contributes to resistance development in bacteria circulating in animal reservoirs. Among them, poultry is one of the main animal reservoirs for the development and dissemination of AMR determinants to the environment and to the food chain. Studies have demonstrated that resistance is increasing for main poultry pathogens over time (265). A study conducted in British Colombia showed that enterococci isolates recovered from poultry litter samples were resistant to lincomycin (LIN), tetracycline (TET), penicillin (PEN), and ciprofloxacin (CIP) in 80.3%, 65.3%, 61.1%, and 49.6%, respectively. Overall more than 80% of the enterococci isolates were resistant to more than one drug tested (133). Studies also showed that there is a high possibility that the MDR of enterococci isolated from animal fecal samples may transfer their resistant determinates to the enterococci found in humans; thus pose a threat to public health (377). Enterococci which were isolated from heart, liver, brain and bone marrow of poultry less than 10 days old were found to have high degree of resistance to sulphamethoxazole/trimethoprim (SXT) (88%), tylosin (TYL) (71.4%), enrofloxacin (ENR) (69.4%), doxycycline (67.3%) and lincomycin (LIN) or spectinomycin (SPE) (56.1%) (51). These findings indicate that poultry and poultry environment are a suitable niche for enterococci AMR development and possible transfer of resistant traits to the other bacterial species in humans or in the environment (106).

1.7 Antimicrobial resistance in *E. coli*

The perseverance of MDR *E. coli* is increasingly observed in human and animal husbandry worldwide. Even though *E.coli* is intrinsically susceptible to most of the clinically important antimicrobials, resistant strains are evolved due to acquisition and dissemination the resistance genes via horizontal gene transfer of mobile genetic elements, including plasmids and transposons. Hence, *E. coli* has already developed resistance to most older antimicrobials including TET, phenicols, SXT and fosfomycin (291). Livestock, more particularly poultry, are considered the main reservoir of pathogenic *E. coli.* The selective pressure exerted by heavy use of antimicrobial agents in livestock promotes the emergence, selection, and dissemination of antimicrobial-resistant

bacteria that circulate in animal hosts and human. According to the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) the prophylactic use of ceftiofur in chickens before voluntary withdrawal in 2014, resulted in extended-spectrum cephalosporin resistance in *E. coli* from chickens and humans in Canada (112). A study conducted in 2017 in China using *E. coli* isolates originating from poultry indicated that 94% of the isolates were at least resistant to one drug. Among them, 83% were MDR. The highest number of isolates were resistant to TET, nalidixic acid, sulfamethoxazole (SSS), SXT, and AMP. An increasing trend of resistance was observed in multiple antimicrobials which are mainly used in human therapeutics including amikacin, aztreonam, ceftazidime, cefotaxime, chloramphenicol (CHL), CIP (394). A recent study conducted in Canada showed that there are genetic similarities between human pathogenic *E. coli* strains isolated from UTI and *E. coli* isolated from chickens in processing plants and retail poultry. The study concluded that poultry could be a reservoir for human pathogenic *E. coli* and raised the possibility of transmitting resistance from poultry-to-human strains. (35). Another study conducted in Canada in order to determine the prevalence of resistance of *E. coli* strains isolated from 2005-2015 inhabiting healthy chickens, more particularly from cecum over a 10 year period, showed that a majority of isolates were susceptible to most of the antimicrobials tested. Among the resistant strains, resistance to third generation cephalosporins and β-lactams with β-lactamase inhibitors were the common finding. MDR was observed in 38.5% of isolates (228). Hence harboring resistance even in commensal strains, is a striking finding and shows a threat to human and animal health. *E.coli* isolates recovered from colibacillosis in Nepal poultry farms showed that 22% of the isolates were MDR, while AMP (98%), co-trimoxazole (90%), and doxycycline (62%) showed the highest resistance. Among the total isolates, 50% demonstrated intermediate resistance to colistin, (which is considered a last-line therapeutic option against MDR Gram-negative bacterial infections). The rapid detection of colistin-resistant *E. coli* strains in poultry meat premises poses a significant threat to public health (114, 252, 343).

1.8 Polymicrobial infections

Bacterial species coexist in communities where some bacterial species produce biofilms and leading to infections in human and animals. Polymicrobial infections are complicated in nature and interactions among bacteria and mechanisms are poorly understood (286). Although bacterial communities living in mucosal surfaces form mutually beneficial interactions with the host while

opportunistic infections lead to detrimental effects on the host (124). The complex nature of polymicrobial infections is attributed by host-pathogen interactions, microbe-microbe interactions, host defenses against pathogens and environmental factors (286). Not only bacteria but also viruses, fungi and protozoa involved in these infections make the disease process complex resulting in treatment difficulties. Bovine respiratory disease complex is one of the classic examples of multiple etiologies attributed to the production of a disease where 13 viruses and 12 bacterial species were identified with the disease (49). The diverse and dynamic nature of microbial communities associated with cystic fibrosis in humans provides another excellent example for polymicrobial infection (329). It is estimated that biofilms are responsible for most of the infections caused by bacteria and fungi. Complex interactions occur in polymicrobial biofilms which increase the virulence and the pathogenicity of the disease thus leading to detrimental effects on the host (19). The formation of these pathogenic communities leads to persistent and chronic infections. Nosocomial bloodstream infections caused by *Candida albicans* associated with *S. aureus* is a good example of biofilm formation associated polymicrobial infections in humans (158).

Although polymicrobial infections are not widely recognized in poultry, multiple etiologies are involved in the majority of poultry diseases. Among them, the avian respiratory system is an ideal niche for a wide array of interactions taking place among normal inhabitants and pathogenic bacteria, fungi, and viruses. The severity of the disease outcome depends on the type of interaction taking place in terms of synergistic or antagonistic relationships. These outcomes navigate the development of respiratory disease complex in poultry. Avian influenza virus, *Pasturella multocia*, and *E. coli* are the main viral and bacterial agents responsible for disease development in the respiratory system (142, 315). Once the virus infects the respiratory epithelium, the alterations in the infected site lead to increased bacterial adherence and colonization, impairment of the phagocytic activity and/or the alteration of the host immune response. Eventually, these co-infections lead to severe disease and increased bacterial density in the respiratory mucosa. Co-infections occur among avian Influenza, infectious bronchitis virus, Newcastle disease Virus, *Staphylococcus* species, *Ornithobacterium rhinotracheale*, *Mycoplasma* species and *E. coli* are responsible for respiratory disease complex in poultry. Increased mortality rates are reported in flocks infected with multiple respiratory pathogens (330). When broilers were challenged with H9N2, a low pathogenic avian influenza along with *E. coli*, the mortality rates were increased

compared to monomicrobial infection. It is postulated that *E. coli* infections are accelerated due to mucosal damage caused by avian influenza virus. Once colonized, *E. coli* causes septicemia and leads to increased mortality in birds. Likewise, the synergistic effects of *E. coli* and *Mycoplasma* tend to increase dissemination of *E. coli* throughout the body and increases the severity of infection (256, 315). Severe enteritis and higher mortalities were observed in young turkeys co-infected with enteropathogenic *E. coli* and turkey coronavirus compared to an infection with a single organism. Attaching/effacing intestinal lesions due to *E. coli* cause extensive damage and persisted in the intestines if birds were co-infected (155). Not only young and old birds, chicken embryos and neonatal chickens are also prone to polymicrobial infections, as the pathogens can invade via both vertical transmission and horizontal transmission from the environment during incubation. These infections may increase embryo and neonatal mortalities. Although the pathogenesis is not clear, isolation of multiple bacterial species from non-viable chicken embryos indicates polymicrobial infections of embryos lead to embryonic death (157). Once embryonated eggs were co-infected with *G. anatis* and *E. coli*, mortality rates were significantly higher compared to a single pathogen (379). To elucidate the pathogenic mechanism of *E. faecalis* and *E. coli,* the following objectives were proposed to study host-pathogen interactions of chicken embryos and neonatal chickens with *E. faecalis* and *E. coli*.

1.9 Objectives

1. Isolation and identification of bacterial species associated with broiler chicken embryo mortality in commercial hatcheries in western Canada
2. Determine the AMR profiles of *Enterococcus* and *E. coli* isolated from non-viable broiler chicken embryos from commercial hatcheries in western Canada
3. Development of a specific pathogen free (SPF) chicken embryo model to study pathogenesis of *E. faecalis* and/or *E. coli*.

Chapter 2 INCREASED INCIDENCE OF ENTEROCOCCAL INFECTIONS IN NON-VIABLE BROILER CHICKEN EMBRYOS IN WESTERN CANADIAN HATCHERIES AS DETECTED BY MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME OF FLIGHT MASS SPECTROMETRY

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Contribution: The research concept was designed by my self and Dr. Gomis. The sample collection was conducted by my self, Shelly Popowich and Morgan Wawryk. The members of the research group assisted to conduct the laboratory experiments. I wrote the manuscript under Dr. Gomis' guidance and all the authors mentioned above contributed with their feedback.

2.1Abstract

Among bacterial infections, enterococci has become an emerging pathogen in the poultry industry worldwide. The objectives of this study were to determine the bacteria associated with non-viable broiler chicken embryos in three western Canadian poultry hatcheries and to determine the effectiveness of matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) for bacterial identification. The results were similar in all three hatcheries for the embryo mortality pattern occurring during incubation and the breakout analysis. The late stage of embryo mortality was the predominant (35.08%) followed by the early stage embryonic death (15.35%). The bacterial isolation from yolk samples showed that 65.82% of swabs were positive for at least one type of bacterial growth while 34.17% of swabs were negative for bacterial isolation. The mixed bacteria populations were observed in 34.3% of the samples positive for bacteria growth. The rest (31.52%) of the samples were only had one type of bacterial growth. The frequency of bacterial isolation from hatch debris (60-75%) increased with the age of broiler breeders. MALDI-TOF MS was able to identify 83.13% of isolated bacteria into genus level. Moreover, MALDI-TOF MS identified *Enterococcus* and *E.coli* isolates with 97.18% and 100% accuracy at species level, respectively. Whereas *Staphylococcus* species were identified with 62.59% accuracy. The congruence between identification obtained from MALDI -TOF MS and 16S rRNA or Chaperone 60 (*cpn60*) sequencing was 100% or 90%, respectively. Of all bacteria isolated, *Enterococcus* species (29.71%) were the most prevalent followed by *E. coli* (19.46%). About 56% of *E. coli* infected samples were co-infected with *Enterococcus* species. Among all *Enterococcus* species isolated, *E. faecalis* (79.58%) was the most prevalent followed by *E. faecium* (8.1%). Overall, our study showed that *Enterococcus*-associated embryo mortality was predominant in all the three hatcheries and it suggests that MALDI-TOF MS platform is an accurate tool for bacterial identification such as *Enterococcus* species, isolated from poultry.

2.2 Introduction

Embryonic death during incubation and first week mortality of broiler chickens can cause significant economic losses to the poultry industry (274, 307, 395). These embryonic deaths can be attributed to infectious or non-infectious agents (307). A variety of bacterial species have been isolated from non-viable chicken embryos at different rates (176, 275). Bacterial contamination can occur at different stages of the broiler breeder production cycle. Some researchers have suggested that bacterial contamination of fertile eggs is higher at the hatchery than at the broiler breeder level (83, 201). The increased incidence of YSI after introduction of commercial hatcheries in 1930s, support the above observation (79). Environmental and management factors influence the introduction of bacteria into hatching eggs. Factors related to introduction of bacteria into hatching eggs include; eggshell pore size, the number of pores, surface area of the eggshell and eggshell thickness (70, 93, 146, 263). The occurrence of spontaneous or handling induced hairline or micro-cracks can also facilitate bacterial contamination of hatching eggs (14, 29, 383). Researchers have noted that bacterial penetration into eggs is high immediately after laying because of the wet cuticle (247). The relationship between the age of the breeders and bacterial penetration has also been extensively studied (181). It was also reported that bacterial penetration can occur at higher rate at the end of lay compared to beginning of the lay (262). Sanitary status of *in ovo* vaccination equipment and vaccine room are other important factors that can facilitate bacterial contamination of developing embryos or the newly hatched chicks. Therefore, *in ovo* vaccination at day 18 of incubation in the hatchery requires strict hygienic practices to reduce microbiological contamination of eggs (385). Marek's vaccine contamination with *E. faecalis* was reported in hatchery environment and possible airborne contamination was postulated (212).

Hatching eggs contamination with *Salmonella* serotypes was extensively studied. Trans ovarian transmission and horizontal transmission of these serotypes into chicken eggs were noticed (170, 246). Among those, *S.* Pullorum, *S.* Gallinarum were able to penetrate the eggshells and multiply within internal compartments of incubating eggs (342). Later, *S.* Pullorum infection was largely controlled in developed countries (30). At present, *S.* Enteritidis became the dominant *Salmonella* serotype species isolated from eggs and laying hens which impose a human health hazard (92, 138). Later, *E. coli* has been reported as the predominant bacterial species responsible for chicken embryo death in poultry hatcheries worldwide (264). Furthermore, *Staphylococcus, Streptococcus, Pseudomonas, Enterobacter, Klebsiella, Salmonella, Proteus, Citrobacter* and

Bacillus species have also been recovered at varying degrees from non-viable chicken embryos and YSI (8, 16, 26, 79, 258, 275, 344).

Recently, the emergence of *Enterococcus* associated first week mortality was reported in many countries around the world including Canada (264). Causes associated with the emergence of enterococcal infections in poultry are not well understood. Apart from the emergence of enterococcal YSI, *Enterococcu*s species were *E. cecorum* associated osteomyelitis and spondylitis considered as an emerging and economically significant infectious disease in the poultry industry (187, 231, 302). *E. faecalis* associated amyloid arthropathy in layers and encephalomalacia associated with recovery of *E. durans* and *E. hirae* in young broiler chickens were also described (3, 58, 64, 211). Although these infections were reproducible, pathogenesis and virulence mechanisms associated with these infections are poorly understood (44, 184).

It is well documented that poor hygienic practices lead to poor chick quality and poor performances in the production cycle (385). Hatching egg contamination is a critical control point in the transmission of bacteria (such as pathogenic *Salmonella* serotypes, *E. coli* and coliforms) to day-old chicks and via the food chain to the consumer at the end (83). Hence, rapid and accurate identification of bacterial species is essential for providing proper remedies in a timely manner to prevent diseases in poultry and to ensure human health (218). The conventional techniques for identification of bacteria are time-consuming and costly and may fail to provide accurate identification of bacteria to species level. Bacterial identification by molecular approaches, such as universal gene targets, is also time-consuming and costly (40). Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) is an increasingly used molecular based techniques enabling rapid identification of bacteria to the species or even sub-species level (95). MALDI -TOF MS analyzes the mass to charge ratio of ionized bacterial protein molecules and produces unique mass spectral finger prints of each bacterial isolates which placed on target plates. The resulting protein spectra are postulated to be characteristic for each bacterial species. Then the obtained profiles are compared to the database of highly selective and specific spectra from reference strains (56). MALDI-TOF MS exhibits a high discriminatory power of identifying bacteria even into strain level. The MALDI Biotyper 2.0 (Bruker Daltonics) is the largest and most expanded efficient spectral database created to date that includes both medical and veterinary data (324). This novel technology is currently used in both medical and veterinary diagnostic laboratories and provides accurate identification of bacterial species with a rapid

turnaround time (57). Conventional biochemical tests sometimes fail to identify bacteria in environmental samples due to their vast diversity (364). MALDI-TOF MS technique has been successfully evaluated for accurately identifying environmental microbes in various ecosystems (125, 313, 366)

Therefore, the objective of this study was to identify bacteria associated with broiler chicken embryo mortality in western Canadian hatcheries by examining hatch debris using the MALDI-TOF MS, a well-accepted and efficient molecular technique for bacterial identification.

2.3 Materials and methods

2.3.1 Collection of non-viable chicken embryos

Unhatched broiler-hatching eggs (hatch debris) at 21 d of incubation were collected from two commercial broiler hatcheries in Saskatchewan and one in Alberta, Canada. Hatch debris was randomly collected at convenient time points and intervals. These unhatched eggs were collected immediately after healthy chicks were removed from hatch trays. A total of 55 broiler breeder flocks were sampled: once (14 flocks), twice (19 flocks), three times (10 flocks), four times (7 flocks) and five times (5 flocks) from November 2013 to August 2014. The age of the broiler breeders ranged from 26-63 weeks of age (median=43 weeks, SD=9.7). Hatchability of the breeders ranged from 47%- 88% (median=81%, SD=9.7). A median of 19 (range 8-169) non-viable eggs (hatch debris) were collected at each sample collection time point per broiler breeder flock. A total of 3,896 hatch debris samples were collected from 15 hatch debris sampling periods (hatchery A = 5 times, hatchery B = 6 times and hatchery C = 4 times) from the 55 broiler breeder flocks during the study. *In ovo* vaccination was performed in hatchery A and hatchery B, but not in hatchery C during our sample collection.

2.3.2 Breakout analysis of unhatched eggs (estimation of age of chicken embryos)

Eggshell and shell membrane were removed from the air cell end of the egg using scissors in order to examine non-viable embryos and contents of the eggs. The estimation of age at death of non-viable chicken embryos (or breakout analysis) was performed according to Pas Reform, HatchTech and Aviagen Inc. guidelines with modifications (25, 156, 335). Non-viable embryos were divided into several categories such as infertile, early-embryonic death (between 1-7 d), mid-

embryonic death (between 8-14 d) and late-embryonic death (between 15-21 d). Infertile category had dense round white spot indicating the blastodisc with no obvious signs of embryo development. Early embryonic death was identified by visible extra embryonic membranes, blood rings, (disintegration of blood vessels) or a small dead embryo with pigmentation. Mid-embryonic death category had embryos with or without an egg tooth but no feather development, and late embryonic death category had fully developed, feathered embryos with or without YS absorption or internal pipping (pipping of the eggshell membrane). External pipping (pipping of the eggshell) was also recorded. Malformations of embryos such as extra limbs, crossed beaks, exposed brains, missing appendages and exposed viscera were recorded. Dark yellowish-brown to black semisolid contents with foul smelling material indicated death of embryos at an undermined age. Fully developed chicks which were viable but unable to hatch at 21 d, were defined as slow. Cracks in egg shells (probably due to mishandling during the transfer of eggs from the incubator to hatcher on 18-d of incubation) were recorded as cracks.

2.3.3 Sample collection and bacterial culture conditions

Egg yolk or contents were collected in a separate clean room away from hatchers in the hatchery. They were aseptically collected near a Bunsen burner to make the immediate environment sterile and yolk was collected using sterile cotton swabs for bacterial culture. As an environment control, a Colombia BA plate was kept open for 15 min to expose to the air and repeated in every sampling day. During the study period, a median of 6 (range 3-20) bacterial swabs per broiler breeder flock were collected for a total of 765 swabs. These swabs were collected from each category of hatch debris [infertile (n=43); early embryonic death (n=177); mid embryonic death (n=112); late embryonic deaths including internal pipping (n=286); malformation (n=54); undetermined age (n=18); external piping (n=22); slow (n=41); and; cracks (n=12)]. Swabs were cultured on 5% Columbia sheep BA (Oxoid, Nepean, Ontario, Canada) and incubated aerobically at 37 C for 24-48 h. Among these, 384 randomly selected swabs were cultured and incubated in anaerobic conditions to examine potential presence of anaerobic bacteria. Briefly, swabs were plated in 5% Columbia sheep BA, placed in an incubation chamber (BD GasPak EZ Container, BD Canada Mississauga, ON) with a an anaerobic gas generating sachet (AnaeroGen Sachet, Oxoid, Nepean, ON) and resazurin anaerobic indicator strip and incubated for 48 h at 37 C. Bacterial growth and colony morphology were recorded from primary growth plates. Growth

on these plates was reported on a scale from 0 to 4+ (153). Mixed cultures were sub-cultured on BA for purity, all pure cultures were stored in brain heart infusion (BHI) broth (Becton, Dickinson & Company, France) supplemented with 20% glycerol (w/v) (Fisher Scientific, Fair lawn, New Jersey) at -80ºC.

2.3.4 Bacterial identification by MALDI-TOF MS

A total of 889 bacterial isolates were processed for identification using MALDI-TOF MS Biotyper (Bruker Daltonik, Bremen, Germany) Compass version 1.4 software, as previously described (372). Briefly, fresh pure bacterial isolates were smeared on the MALDI-TOF MS target plate with a thin film of bacteria, overlaid with 1 µL of matrix solution "α-cyano-4-hydroxycinnamic acid" referred to as direct transfer method. Thereafter, samples were co-crystallized by air drying at room temperature. Mass spectra were obtained by MALDI Biotyper, Compass version 1.4 software which includes Flex Control software for spectra acquisition and Real Time Classification software which matches acquired spectra to Bruker's main library which currently has spectra for more than 5600 bacteria of human and veterinary origin and provides corresponding possible identification. The bacterial test standard was applied to every target for each run. Therefore, each run was calibrated using the bacterial test standard. The formic acid extraction method was performed as previously described for isolates when no reliable identification was obtained (39). According to the Bruker recommendations, the level of similarity between an unknown bacterial isolate and a reference strain is indicated by a log (score). If the score > 2 indicates secure species level identification, a score between ≥1.7 and < 2 indicates a secure genus identification and a score < 1.7 indicates a non-reliable identification.

2.3.5 Bacterial identification by 16S rRNA and Chaperone 60 *(cpn60)* gene sequencing

When both the direct transfer and formic acid extraction methods failed to provide a reliable identification or provided identification only at genus level, 16S rRNA gene sequencing and *cpn60* universal gene sequencing were performed for randomly selected 40 isolates (25 isolates with no reliable identification, 10 *Staphylococcus*, 3 *Enterococcus*, 2 *Salmonella*).

Another group of 20 isolates with good species identification using MALDI-TOF MS (*i.e.* two isolates of *E. faecalis*, *E. faecium*, *E. casselflavus*, *E. coli*, and single isolates of *E.gallinarum*,

E. avium, *S. aureus*, *S. epidermidis*, *S. simulans*, *S. hycius*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *P. mendocina*, *Bacillus cereus*, *B. pumillus* and *Acinetobacter baumanii*) were selected and subjected to *cpn60* and 16S rRNA gene sequencing to compare the bacterial identity between MALDI-TOF MS, *cpn60* or 16S rRNA techniques. Furthermore, American Type Culture Collection (ATCC) strains (*E. faecalis* ATCC 29212, *E. coli* 25922, *B. cereus* 10876 and *S. aureus* 25923) were used as positive controls. All the isolates above were grown on 5% Columbia sheep BA at 37ºC for 48 h. Genomic DNA of these isolates were extracted using boiling method (10). PCR was performed to amplify the 500 bp fragment of 16S rRNA gene (375) and 700 bp fragment of the c*pn60* gene as described previously (145).

2.4 Statistical analysis

Descriptive statistics were calculated for each category of incubation following breakout analysis of unhatched eggs using Excel 2010. The independence of association between pairs of variables was tested using the Chi-square test (Statistix 7, Tallahassee, FL).

2.5 Results

2.5.1 Breakout analysis and embryo mortality pattern

Breakout analyses confirmed the highest embryo mortality occurred during the late stage of incubation (n=1,393 embryos or 35.08%) followed by the early stage (n=598 embryos or 15.35%). The third highest category was undetermined age (n=517 eggs or 13.27%). The incidence of the remainder of the breakout categories in descending order were due to infertile eggs (n=387 eggs or 9.93%), slow embryos (n=385 embryos or 9.88%), malformations (n=308 embryos or 7.91%), mid stage (n=133 embryos or 3.41%), external piping (n=115 embryos or 2.95%, and cracks (n=60 eggs or 1.54%). The frequency of these breakout categories in each of the three hatcheries are showed in Figure 2. 1.

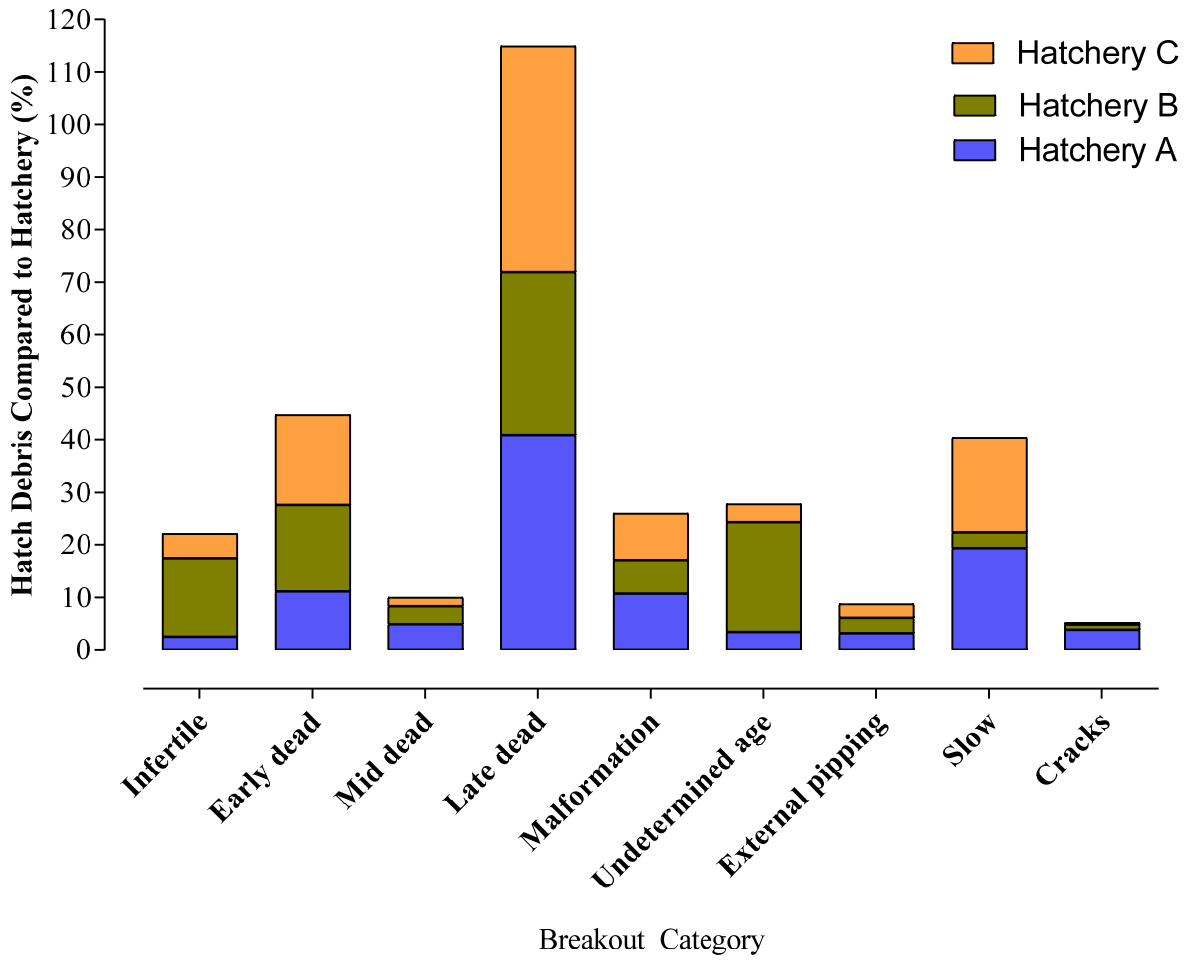

Figure 2.1: Comparison of categories of breakout analysis of broiler chicken embryos according to the hatchery . [infertile (no obvious indication of embryo development); early dead (between 0-7 d); mid dead (between 8-14 d); late dead (between 15-21 d) including internal pipping; malformations (extra limbs, cross beak, exposed brain and exposed viscera); undetermined age (dark yellowish-brown and black semisolid contents with foul smelling material indicated); external pipping (pipping of the egg shell); slow (fully developed chick but unable to pip at the day of hatch); cracks (due to mishandling of eggs during transferring of eggs from the incubator to hatcher on 18-d of incubation)] were also recorded.

The percentage of breakout categories according to breeder age is illustrated in Figure 2. 2. Breeder age was categorized as young (<34 weeks), prime (34-49 weeks) and old (>49 weeks) (156). The young breeders had highest percentages of late embryo death (42.47 %), slow (21.14%), mid stage (8.81%) and external pipping (3.33%). Prime breeders had highest percentages of early stage (18.14%), malformations (9.13%) and mid stage embryo death (3.14%). Undetermined ages (25.05%) and infertile (11.59%) stages were highest in old breeders. The prevalence of cracks were quite similar in all the age groups. The frequency of bacterial isolation among categories of

hatch debris from highest to lowest rate is as follows; undermined age (100%), external piping (77.27%), mid embryo death (69.64%), malformation (66.67%), cracks (66.67%), late embryo death (64.33%), early embryo death (63.84%), slow (60.97%) and infertile (53.49%).

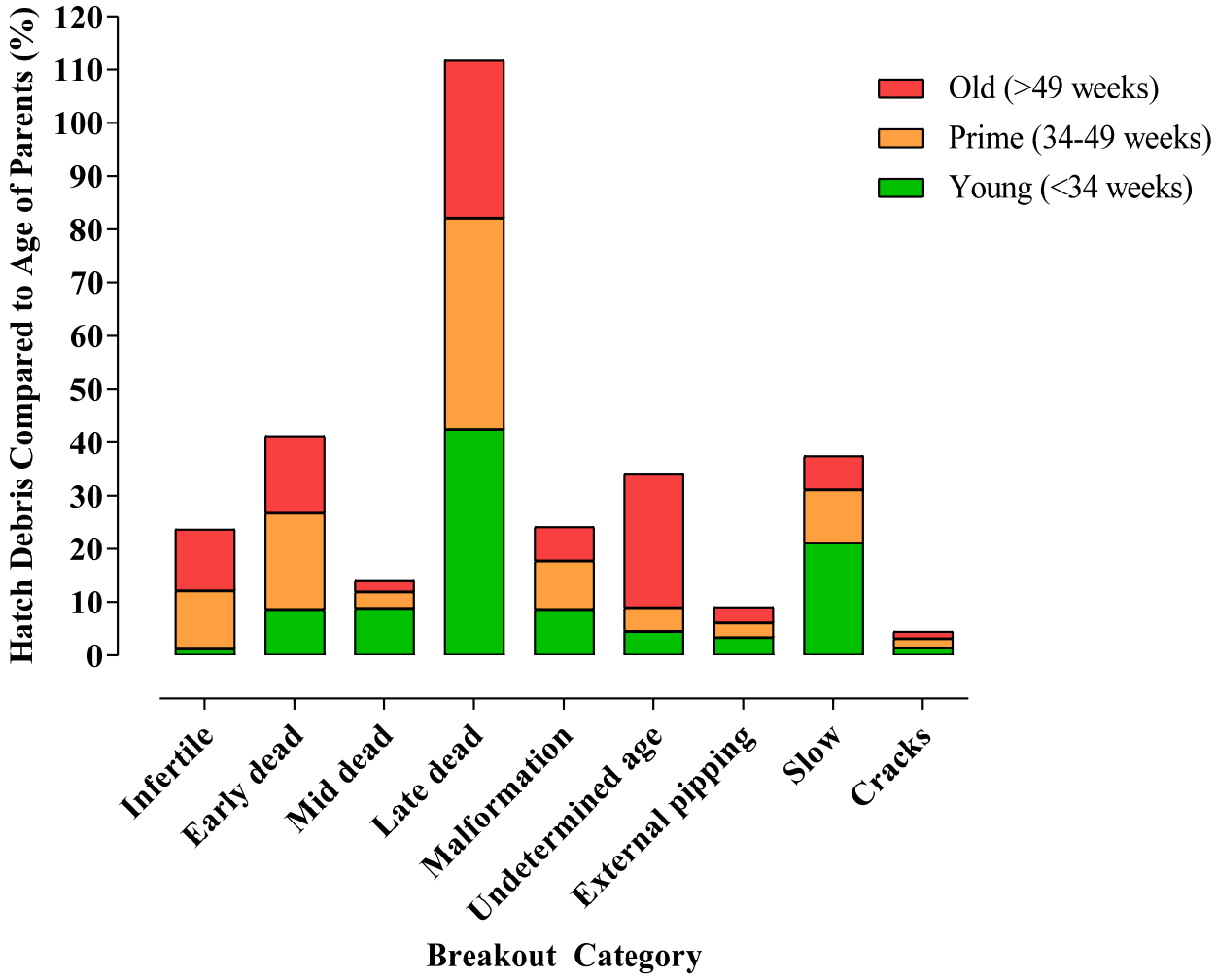

Figure 2.2: Comparison of categories of breakout analysis of broiler chicken embryos according to the age of broiler breeder parents. young (<34 weeks); prime (34-49 weeks) and old (>49 weeks) [infertile (no obvious indication of embryo development); early dead (between 0-7 d); mid dead (between 8-14 d); late dead (between 15-21 d) including internal pipping; malformations (extra limbs, cross beak, exposed brain and exposed viscera); undetermined age (dark yellowish-brown and black semisolid contents with foul smelling material indicated); external pipping (pipping of the egg shell); slow (fully developed chick but unable to pip at the day of hatch); cracks (due to mishandling of eggs during transferring of eggs from the incubator to hatcher on 18-d of incubation)] were also recorded. Isolation of bacteria from non-viable chicken embryos or contents of unhatched eggs.

A total of 956 bacterial isolates were recovered from the 765 swabs collected. The breakout analysis of unhatched eggs showed that 65.82% of swabs obtained from yolk samples had at least one type of bacterial growth while 34.17% of swabs were negative. Of those 65.82% swabs with bacterial growth, 34.3% swabs yielded mixed cultures while 31.52 % yielded pure culture. The rate of bacterial isolation varied from 60-75% depending on the age of broiler breeders (Figure 2. 3) Chi-square analysis shows that the greater isolation of bacteria from hatch debris of older breeders is unlikely to have occurred by chance ($p < 0.01$). Also, among the samples from which bacteria were recovered, as the age of broiler breeders increased, pure cultures were more frequent. Chi-square analysis shows that the greater isolation of a pure cultures of bacteria from hatch debris of older breeders is unlikely to have occurred by chance ($p < 0.05$). No strict anaerobic bacteria were isolated from any of the hatch debris samples.

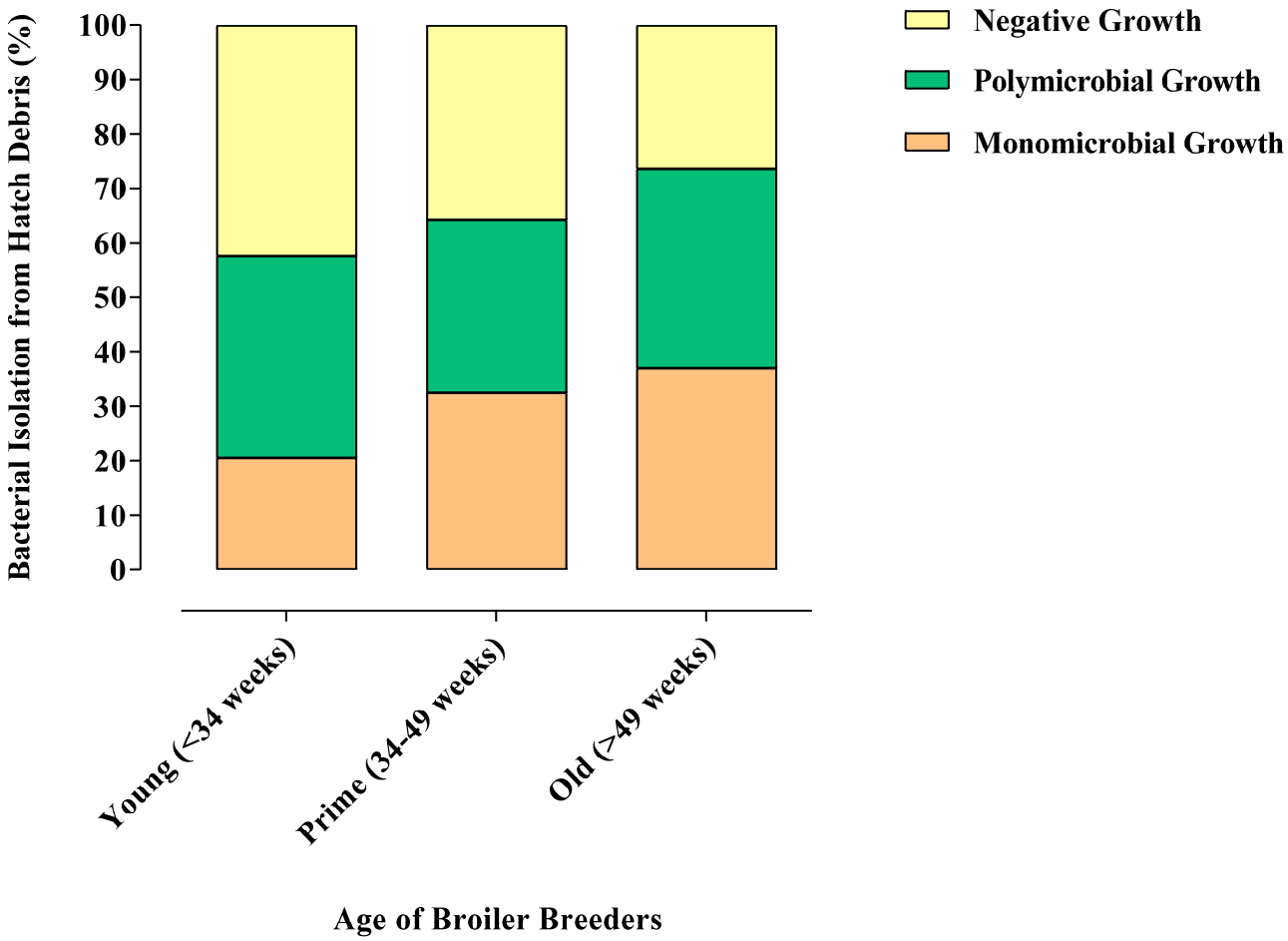

Figure 2.3: Comparison of bacteria isolation rate as swabs taken from hatch debris according to the age of broiler breeders. [*i.e.* pure culture (monomicrobial) or multiple colony type (polymicrobial) for different species of bacteria from hatch debris].

2.5.2 Bacterial identification using MALDI-TOF MS

MALDI-TOF MS was able to identify bacteria at the genus level in 83.13% (739/889) of isolates and species level in 67.15% (597/889) of isolates. Bacterial species of *Enterobacteriaceae*, non-fermentative Gram-negative, and Gram-positive were identified to the species level using MALDI-TOF MS (Table 2. 1)

Table 2.1: Identification of different bacterial species by MALDI-TOF MS. Seven hundred and thirty nine of 889 (83.13%) bacterial isolates from hatch debris were identified by MALDI-TOF MS and the remaining 150 (16.87%) isolates were not able to identify.

Number of bacterial isolates identified by MALDI-TOF MS	Percentage of bacteria identified up to the species level by MALDI-TOF MS
Enterobacteriaceae (n=218) *Citrobacter* species, *Enterobacter* species, *E. coli*, *Klebsiella* species, *Salmonella* species, *Pantoea* species, *Providentia* species, *Serratia* species	**90.82%** **(198 of 218 isolates)**
Non fermentative Gram-negative bacteria (n=32) *Acinetobacter* species, *Pseudomonas* species, *Stenotrophomonas* species	**84.37%** **(27 of 32 isolates)**
Gram-positive cocci (n=435) *Enterococcus* species, *Globicatella* species, *Micrococcus* species, *Staphylococcus* species, *Streptococcus* species, *Vagococcus* species	**81.38%** **(354 of 435 isolates)**
Miscellaneous bacteria (n=54) *Aeromonas* species, *Bacillus* species, *Corynebacteriu*m species, *Neisseria* species, *Rothia* species	**68.52%** **(37 of 54 isolates)**

MALDI-TOF MS analysis revealed that the majority of non-viable eggs had *Enterococcus* species (29.71 %) followed by *E. coli* (19.46 %). Other bacterial species isolated included *Staphylococcus* species (14.54%), *Proteus* species (7.0%), *Bacillus* species (3.56%), *Salmonella* species (1.46%) and *Pseudomonas* species (1.36 %). Miscellaneous bacterial isolates less than 1% for each included *Enterobacter* species, *Streptococcus* species, *Klebsiella* species, *Corynebacterium* species, *Acinetobacter* species, *Aeromonas* species, *Citrobacter* species, *Globicatella* species, *Micrococcus* species, *Neisseria* species, *Pantoea* species*, Providencia* species, *Rothia* species, *Serratia* species, *Stenotrophomonas* species and *Vagococcus* species. Bacterial isolates with no reliable identifications accounted for 15.7% of isolates (Figure 2. 4). MALDI-TOF MS identified *Enterococcus, E.coli,* and *Staphylococcus* isolates with 97.18%, 100% and 62.59% accuracy at the species level, respectively. The rate of isolation of top three

bacterial species (*i.e. Enterococcus* species, *E.coli* and *Staphylococcus* species) followed a similar pattern among all three hatcheries (Figure 2. 5). Although, *Salmonella* species were isolated from hatch debris, it was restricted to one hatchery (1.5%) (Figure 2. 5).

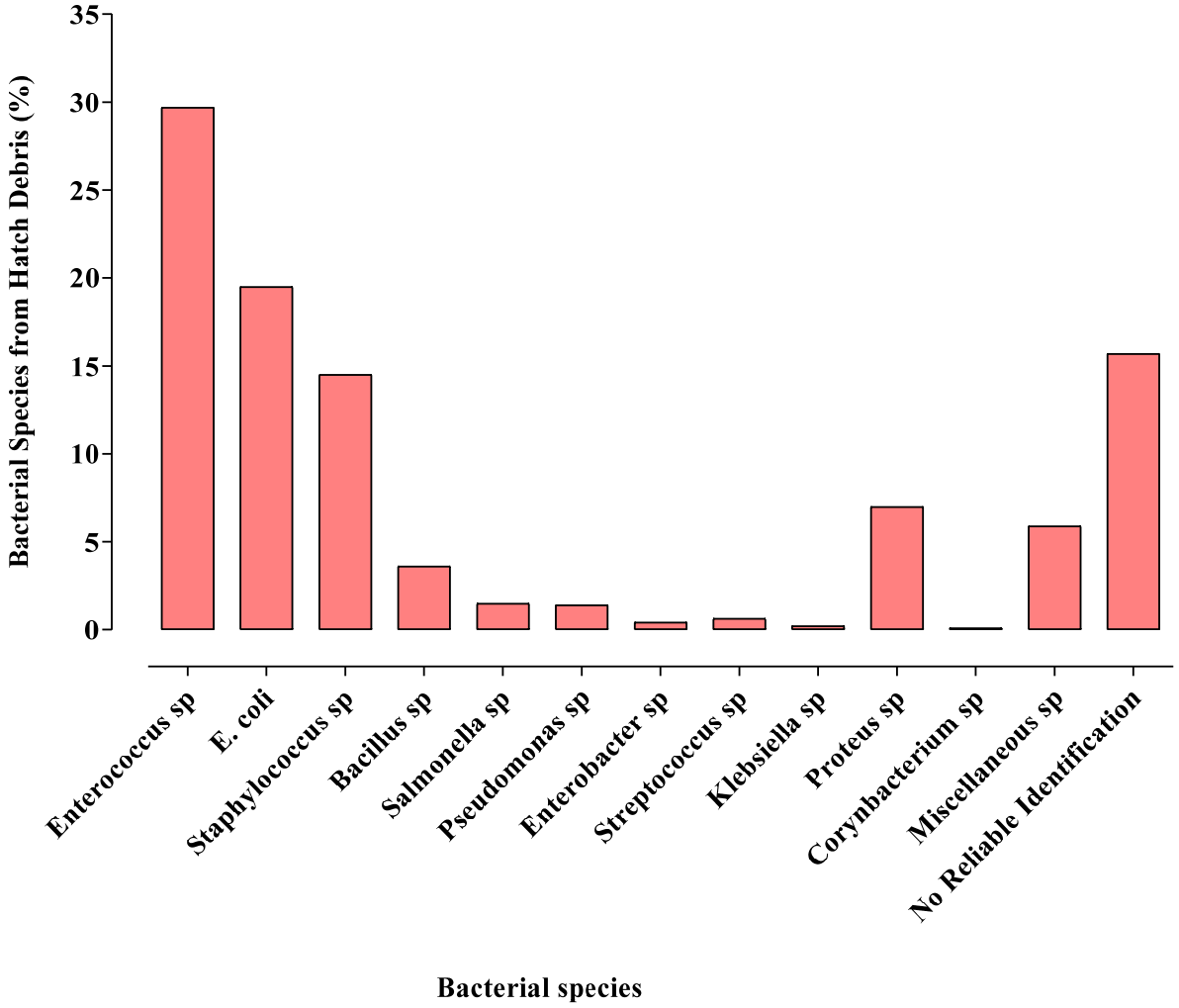

Figure 2.4: Rate of isolation of bacterial species from hatch debris. [Miscellaneous species: *Acinetobacter* species, *Aeromonas* species, *Citrobacter* species, *Globicatella* species, *Micrococcus* species, *Neisseria* species, *Pantoea* species, *Providencia* species, *Rothia* species, *Serratia* species, *Stenotrophomonas* species and *Vagococcus* species; identified by MALDI-TOF MS].

Isolation rates of *Enterococcus* species and *E. coli* according to breeder age is indicated in Figure 2. 6. There was no significant association between species or type of bacteria and breeder age ($p > 0.05$). *Enterococcus* species and *E. coli* were present more frequently as polymicrobial growth (mixed culture) than monomicrobial growth (pure culture). Majority of *Enterococcus* and

E. coli isolates were recovered as polymicrobial cultures than pure cultures. Co-isolation rate of *Enterococcus* species with *E. coli* was 56.63%.

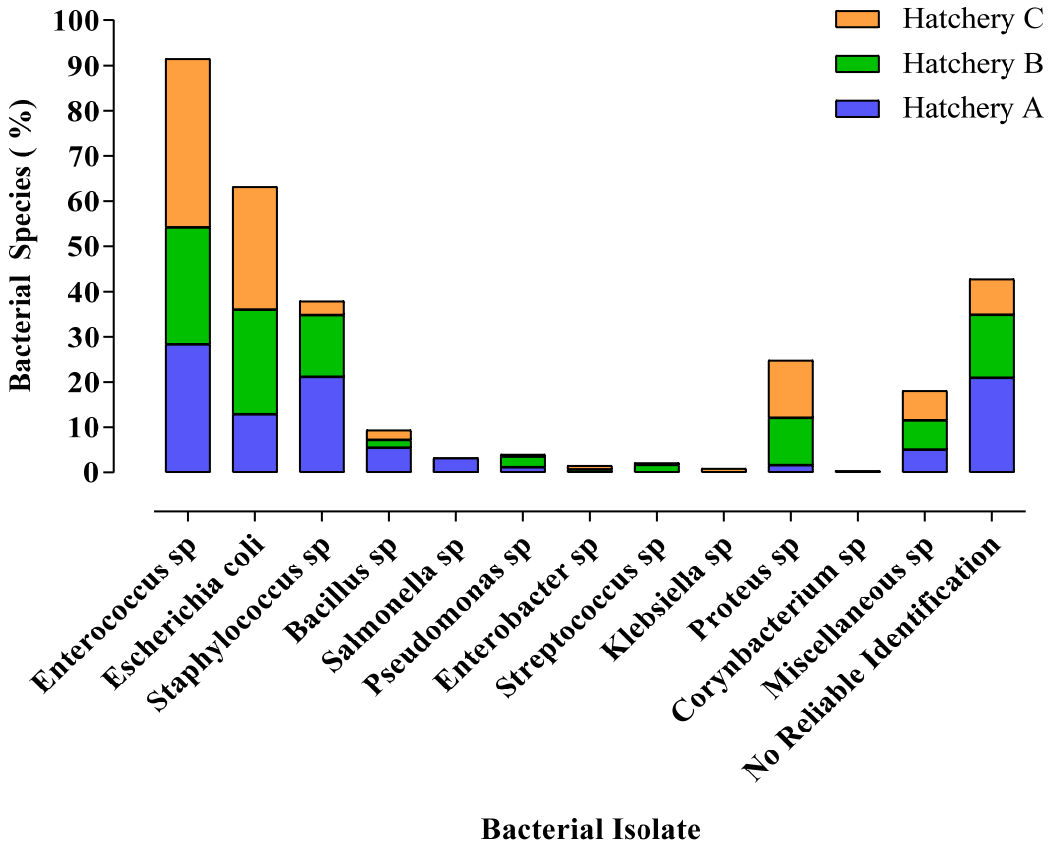

Figure 2.5: Comparison of bacterial species from hatch debris among three hatcheries.

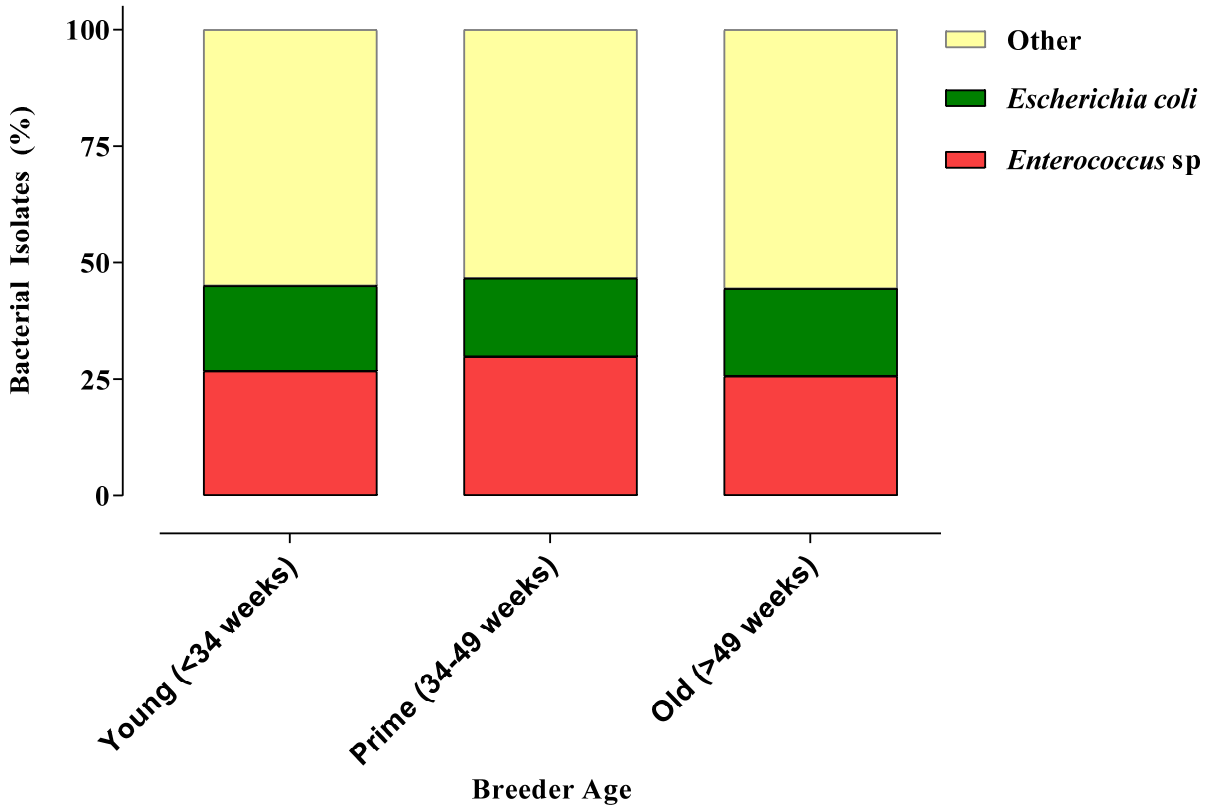

Figure 2.6: Comparison of percentage of *Enterococcus* species, *E. coli* and other bacterial species from hatch debris according the age of broiler breeders.

The predominant *Enterococcus* species isolated was *E. faecalis* (79.6%), followed by *E. faecium* (8.1%) in all three hatcheries. The most common *Enterococcus* species were *E. faecalis*, *E. faecium* and *E. gallinarum*. They were isolated from hatcheries A (66.5%, 10.9% and 3.4%), B (75.6%, 7.3% and 7.3%) and C (88.0%, 4.8% and 1.2%) respectively. *E. avium* (3.9%) and *E. casseliflavus* (1.1%) were also isolated. MALDI-TOF MS identified 3.5% of *Enterococcus* isolates only to the genus level.

Of all isolates, 16% (150/889) had no reliable identification either from direct transfer method or formic acid extraction method. From 25 of 40 randomly selected isolates with no reliable identification, 76 % (19/25) were identified as *Staphylococcus* species. *cpn60* gene sequencing was able to identify these 19 *Staphylococcus* isolates to species level (100%) while 16S rRNA identified them only at the genus level. These *Staphylococcus* species included *S. pulverei*, *S. simulans*, *S. vitulinus*, *S. sciuri*, *S. epidermidis* and S. *fleuretti*. The remaining 6/25

isolates with no reliable identification were identified as *Actinomyces nasicola* (3/6), *Brevibacterium luteolum* (2/6), and *Brevibacterium species* (1/6). 15/40 isolates with only genus level identifications were distributed among *S. pulverei* (6/15), *S. simulans* (2/15), *S. sciuri* (1/15), *S. epidermidis* (1/15), *S. vitulinus* (1/15), *E. faecalis* (1/15), *E. gallinarum* (1/15) *and Salmonella enterica* subspecies *enterica* (2/15).

2.5.3 Comparison of MALDI-TOF MS and gene sequencing methods for bacterial identification

From the 20 selected isolates initially identified using MALDI-TOF MS, there was a 100% (20/20 isolates) match between MALDI-TOF MS and 16S rRNA gene sequencing analysis and 90% (18/20 isolates) concordance between MALDI-TOF MS and *cpn60* gene sequencing.

2.6 Discussion

Good broiler breeder and hatchery management practices are very important for production of good quality day-old chicks (360). Both poor broiler breeder and hatchery management practices may lead to embryo morality and YSI (395). Unsanitary conditions of hatcheries may lead to bacterial infection of neonatal chicks through unhealed navels and cause YSI (200). Bacteria may also enter hatching eggs due to contamination of egg shells with feces in broiler breeder farms or due to vertical transmission of certain bacterial species (in particular *Salmonella* species) from broiler breeder parents to their progeny (162). YSI result in increased neonatal broiler chicken mortality, chronic infections, increased culling and condemnations, which results in significant economic losses to the poultry industry (16). The pattern of embryonic mortality during incubation provides useful information on management practices (177). As we have seen in this study, late embryo death was the major category associated with embryo mortality followed by early embryo death, in all three hatcheries. A higher incidence of early and late embryo mortality is well documented and may be due to factors such as rapid embryonic development, breeder management, egg storage and incubation conditions (208). This pattern of embryo mortality was consistent with previous observations (96, 359).

In the present study, we found an increased isolation of both monomicrobial and polymicrobial cultures from hatch debris with the progression of the age of broiler breeders. Penetration of bacteria through the egg shell or transmigration of bacteria is higher when egg shell

quality is decreased with the progression of the age of broiler breeders (181). Increased incidence of *S.* Typhimurium was observed in eggs laid from older breeders as compared to young breeders (38). As expected, we have also seen an increased incidence of infertility in hatching eggs from older breeders in this study as in previous studies (15, 121).

In this study, *Enterococcus* species were the predominant bacterial species isolated from dead embryos followed by *E. coli*, to our knowledge, this has not been reported before. In a previous study, *E. coli* accounted for the majority of embryo deaths compared to *Enterococcus* species (27). Recently, the incidence of *Enterococcus*-associated YSI has been emerging in the poultry industry worldwide, including in Canada (274). We hypothesize that increased incidence of *Enterococcus*-associated embryo mortality predisposes chickens to the increased incidence of *Enterococcus*- associated YSI. This hypothesis is also supported by the recent reports of increased incidence of *Enterococcus*-associated YSI in the commercial broiler industry in western Canada (personal communication, Prairie Diagnostic Services, clinical bacteriology lab; unpublished data). Although *Enterococcus* species were isolated from the majority of samples in this study, further studies are needed to confirm their virulence and reproduction of embryo mortality under experimental conditions.

E. faecalis was the most common bacterial species associated with hatch debris in this study. *E. faecalis* associated amyloid arthropathy in broiler breeders has been reported previously (213). *E. faecalis* is one of the most common pathogens associated with nosocomial infections in human patients (115). The third most common bacterial species recovered in this study were *Staphylococcus* species. Although *Staphylococcus* is considered as a common contaminant, some studies have isolated *Staphylococcus* from dead embryos and cases of YSI. However, the significance of the isolation of *Staphylococcus* species is uncertain (299, 369, 401).

In this study, both *Enterococcus* species and *E. coli* were isolated as mixed cultures more often than in pure culture. It is possible that coinfection or synergism between *Enterococcus* species and *E. coli* may increase embryo mortality, but further experimental studies are needed to prove this hypothesis. Several previous studies in human and other animals suggest the existence of *Enterococcus and E. coli* or other bacterial coinfection or synergism (132, 135, 223). *Enterococcus* species were isolated from polymicrobial infections of soft tissue, peritoneal cavity, cardiac valves, UTI and biliary duct infections in humans (132, 223). Frequent identification of *Enterococcus* species with other facultative anaerobic bacteria from cases of bacteremia in human

patients has also been reported, suggesting synergism of *Enterococcus* with other bacterial species (135). Additionally, cutaneous infections of rabbits with coinfection of *E. coli* and *Enterococcus* developed significantly large cutaneous lesions. These data further support a possible synergistic relationship between *Enterococcus* and *E. coli* (132).

Recent developments in molecular biological techniques such as real-time PCR, universal target gene sequence and microarray provide assistance in identification of bacteria for diagnostic labs to a certain degree but these labs need accurate, cost effective and rapid bacterial identification systems in order to provide antimicrobial therapy to patients in a timely manner (372). MALDI-TOF MS is an alternative to conventional biochemical techniques and molecular based methods which is very cost effective and has a rapid turnaround time (350). In this study, MALDI-TOF MS identified *Enterococcus* with 97.18% accuracy at species level from poultry isolates. During the review process of this manuscript, a study published by Stępień-Pyśniak et al reported that MALDI-TOF MS can identify *Enterococcus* species from wild birds with 94.4% accuracy (341). A recent study conducted to identify poultry isolates of *Lactobacillus* showed that MALDI-TOF MS can identify *Lactobacillus* bacteria with 83.75% accuracy to the species level (95). Moreover, a study conducted in dairy cattle identifying bacteria in milk and faeces, MALDI-TOF MS was able to identify bacteria in the Enterobacteriaceae family to the species level with 92.9% accuracy (305). Furthermore, study conducted using 1,660 clinical isolates from human patients, MALDI-TOF MS was able to identify bacteria to the genus level in 95.4% isolates and to the species level in 84.5% (325). MALDI-TOF MS has been shown to identify *Enterococcus* species (*i.e. E. faecalis, E. faecium*, *E. casseliflavus* and *E. hirae*) with high accuracy (95.7%) from human isolates (280). Our present study in broiler chicken and a recent study in wild birds (341) identified *Enterococcus* species with 97.18% and 94.4% accuracy, respectively, suggesting that MALDI-TOF MS is a very efficient and accurate molecular tool for *Enterococcus* species identification from poultry samples. In contrast, commercially available bacterial isolation kits using phenotypic and biochemical tests provide only presumptive identification of *Enterococcus* species and further molecular based methods are needed to confirm the identification of *Enterococcus,* especially to the species level. This multi-step approach leads to a delay in patient care in addition to additional costs for routine bacterial identification systems in diagnostic laboratories (378).

All of these previous reports confirmed that MALDI-TOF MS can be used as an alternative to conventional biochemical tests such as Analytical Profile Index and other molecular based

approaches including PCR, gene sequencing, and microarray. Our data confirmed that MALDI-TOF MS is a robust and reliable tool for identifying bacteria isolated from non-viable chicken embryos. We have also confirmed 100% agreement of bacterial species between MALDI-TOF MS and 16S rRNA universal target sequencing in this study although using a small number of selected bacterial isolates (20 isolates). In previous literature there was a 97.5% concordance between MALDI-TOF MS and 16S rRNA universal target sequencing for identifying lactobacilli isolated from human fecal and vaginal samples (129).

However, in the present study, MALDI-TOF MS was able to identify *Staphylococcus* isolates with only 62.59% accuracy at the species level. When we performed *cpn60* gene sequencing of the isolates having no reliable identity on MALDI-TOF MS analysis, 76% were identified as *Staphylococcus* species (*S. pulverei*, *S. simulans*, *S. vitulinus*, *S. sciuri*, *S. epidermidis* and S. *fleuretti*). Previous studies on poultry isolates have reported *S. sciuri* and *S. epidermidis* identification using MALDI-TOF MS (42, 165). These data suggest that present MALDI-TOF database can be further improved by adding poultry specific data. Moreover, it was reported recently that even MALDI-TOF MS cannot differentiate *Avibacterium* genus at the species level (12). The effectiveness and robustness of bacterial identification depends on the quality of the reference database. Since there are enormous differences between different strains of bacteria in each bacterial species, quality of the reference database should be high to discriminate each bacterial species (43). Enrichment of MALDI-TOF MS spectral database with poultry specific data will enhance the robustness of MALDI-TOF MS technique in poultry diagnostics.

In conclusion, *Enterococcus* species were the predominant bacteria isolated from hatch debris followed by *E. coli* in three hatcheries in western Canada. The pattern of embryo mortality in these hatcheries suggests an association of embryo mortality, infectious etiology, and hatchery management practices. By identifying possible bacterial etiologies which can increase embryo deaths during the incubation period, we can implement control measures to minimize bacterial contamination from breeder level to the end of incubation period. This study also suggests that MALDI-TOF MS is a reliable and quick method for bacterial identification from poultry that can be further improved by enrichment of poultry specific bacterial spectral database.

PREFACE TO CHAPTER 3

We have demonstrated in Chapter 2 that *Enterococcus* species is the predominant bacterial species associated with broiler chicken embryo mortality in western Canadian hatcheries. Among those, *E. faecalis* accounted for the majority of deaths followed by *E. coli.* The most significant finding in Chapter 2 was co-infection rate of enterococci and *E. coli* in chicken embryos. We found that 56% of embryos were co-infected with *Enterococcus* species and *E. coli*. It is important to note that *E. faecalis* and *E. coli* associated YSI and septicemias in neonatal chickens have increased in the recent past, but the reasons associated with coinfection of bacteria in YSI is not known (198, 203). These infections in the poultry industry are economically important amid reduction of antimicrobial therapeutics (166). It is important to note that antimicrobials resistance in enterococci and *E. coli* are rising and poultry may harbor and disseminate AMR bacteria to the environment and humans via the food chain (54). Hence, it is important to understand the nature of AMR of enterococci and *E. coli* in chicken embryos.

Enterococci are becoming an increasingly crucial human pathogen associated with increased nosocomial infections and increased mortality in debilitated patients due to infections with MDR strains. Some of the hospital adapted strains are resistant to vancomycin, one of the last resort antimicrobials to treat severe bacterial infections (362). *E. coli* strains which carry resistant determinants cause UTI and become a global threat (11).

Pathogenicity of bacteria correlates with carrying AMR genes in their genome (32). Since pathogenic mechanisms of enterococci and *E. coli* are not clear in poultry, identification of AMR patterns of enterococci and *E. coli* is a priority. Because of these reasons, AMR profiles of enterococci and *E. coli* isolated from non- viable chicken embryos described in the Chapter 2 will be studied in Chapter 3.

Chapter 3 NON-VIABLE CHICKEN EMBRYOS: AN OVERLOOKED NICHE HARBORING A SIGNIFICANT SOURCE OF MDR BACTERIA IN THE POULTRY PRODUCTION

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3.1 Abstract

AMR is a global issue, posing a serious threat to public, animal, and environmental health. AMR surveillance at the level of the hatchery is crucial, as it reveals the AMR that would be transferred to the farms and ultimately to humans via the poultry products. Contaminated eggs explode during incubation and contaminate other healthy eggs in the incubator serving as an important source of AMR dissemination. However, there is a knowledge gap regarding the AMR status of bacteria isolated from non-viable chicken embryos. The objective of this study was to investigate the AMR profiles of non-viable chicken embryos focusing on *E. coli* and *Enterococcus* species, which are known to cause infections refractory to antibiotics. *E. coli* (n=170) and *Enterococcus* (n=256) isolates were recovered from yolk material of non-viable broiler chicken embryos at hatch (21 days of incubation), from three commercial hatcheries in western Canada. Antimicrobial susceptibility testing was performed using the Kirby–Bauer disk diffusion method. *E. coli* isolates were resistant (R) to TET, AMP, amoxicillin-clavulanic acid (AMX), SSS, ceftiofur (CEF), gentamycin (GEN) and SPE at the rate of 54.4%, 50.9%, 42.1% 31.7%, 29.8%, 28.6%, 20.5% respectively. Among those, 34.1% of *E. coli* were multidrug-drug resistant. The descending order of AMR of *E. faecalis* was; TET (73.1%), CEF (47.9%), bacitracin (BAC) (43.9%), erythromycin (ERY) (31.4%) and tylosin (TYL) (30.5%). MDR was detected in 35.9% of *E. faecalis* isolates, and 85.7% of *E. faecium* isolates. The most common resistant phenotype of *E. faecalis* was TET (R) + BAC (R) while the most common phenotype of *E. faecium* was CEF (R) + NEO (R) + TET (R) + SXT (R) + PEN (R). To the best of our knowledge, this is the first report on AMR surveillance of non-viable chicken embryos. Overall, the present study revealed that non-viable chicken embryos, an overlooked niche for AMR surveillance, harbor MDR *E. coli,* and enterococci species that can be a substantial source of superbugs in the environment. Our data also highlight the urgency of including non-viable chicken embryos in AMR surveillance programs to understand AMR dissemination and its control.

3.2 Introduction

AMR has become a serious threat to public, animal and environmental health (71, 278). AMR control is a global priority and the World Health Organization has initiated a global action plan to mitigate the emergence and dissemination of AMR (71, 278).The emergence of AMR is multifactorial and may include indiscriminate antimicrobial use and resistance gene transfer from one organism to another. Inappropriate and excessive antimicrobial use in farm animals has been suggested as one of the major causes of the emergence of multidrug-resistant superbugs (161). Consumer awareness about the antimicrobial use in farm-animals and the potential of AMR development is dictating a trend of an increased market demand for organic and antibiotics-free animal products (371).

The European Union banned the VAN analogue, avoparcin, in 1997 and BAC, spiramycin, TYL, and virginiamycin in 1999 for the purpose of prophylactic use in farm animals including in poultry feed (60, 62). Although a reduction of VRE was observed in poultry products in the European Union following the ban on avoparcin since 1997, there has been no reduction of VRE observed in humans (60). Moreover, the fluoroquinolone has been banned in the USA since 2006 for therapeutic use in the poultry industry, but it did not result in a reduction of CIP resistant *Campylobacter* in poultry products (261). Because of these complexities and poor understanding of AMR, concerted efforts are required to identify the potential sources of AMR in a variety of agricultural settings to develop an appropriate control measures (363).

Although, there is no direct evidence available, literature suggests that poultry is a potential source of AMR transmission to humans (34, 172). In commercial poultry production, AMR development and dissemination can occur at several stages of production, such as, at breeder level, at hatchery and at the production farm level. Most of the data on AMR in poultry were generated from the production farms (1, 373) or from the retail poultry meat (91). In the poultry industry, commercial hatcheries act as a link between breeder farms and the production farms. Recent studies suggest that the hatchery is a potential reservoir for AMR bacteria (277) and day-old chicks are a potential source of AMR in chicken farms (255). The comparison of AMR data generated from hatchery samples versus AMR data obtained from poultry farms at the end of production cycle may provide important clues regarding AMR development and its dissemination in the poultry industry (180). The bacterial contamination of hatching eggs can occur at breeder farm level, egg transport and storage, and at hatchery level (61, 90). Bacterial contamination of

developing chicken embryos in hatcheries occurs in many possible ways including contamination of eggshells and penetration of bacteria via cracks in the eggshell, or due to thin eggshells (69, 278). Transmission of bacteria from hatching eggs to their progeny has been demonstrated for bacterial species such as *Campylobacter* and *Salmonella* (224, 312). Most of the studies related to AMR surveillance at the hatchery level have profiled fluff-derived bacteria (402) or day-old chicks (180). Contaminated eggs explode during incubation (168), which may facilitate dissemination of AMR from dead embryos to healthy live embryos and ultimately reaching to humans through contaminated poultry. The contaminated non-viable chicken embryos have been an overlooked niche for AMR surveillance.

Our recent study revealed that the majority of non-viable broiler chicken embryos examined in western Canadian hatcheries were co-infected with *Enterococcus* species and *E. coli* (193). *Enterococcus* species and *E. coli* colonizing the gut of animals are used as bacterial indicators to monitor the prevalence and dissemination of AMR between food animal species and humans (370). Moreover, *E. coli* and *Enterococcus* species can cause significant economic loses to the poultry industry (6, 186). Hence, the present study was designed to fill the knowledge gap by investigating AMR of non-viable chicken embryo using clinical microbiology technique (394). To the best of our knowledge, this is the first report on AMR surveillance on non-viable chicken embryos in hatcheries.

3.3 Materials and methods

3.3.1 Bacterial isolates

E. coli (n=170) and *Enterococcus* (n=256) isolates *i.e.* [*E. faecalis* (n=223), *E. faecium (*n=21), *E. avium* (n=5), *E. gallinarum* (n=5) and *E. casseliflavus* (n=2)] were recovered from yolk material of non-viable broiler chicken embryos at hatch (21 days of incubation), from three commercial broiler hatcheries in western Canada during 2013 and 2014 (194). Bacterial swabs were cultured on 5% Columbia sheep BA (Oxoid Company, Napean, ON) and bacterial identification was done by MALDI-TOF MS (Bruker Daltonics, Milton, ON) as previously described (372). Bacterial isolates were stored in BHI broth (DIFCO®, Detroit, MI) containing 20% glycerol (Thermo Fisher Scientific, Waltham, MA) at -80 C for further studies.

3.3.2 Antimicrobial susceptibility testing

Each bacterial isolate was streaked on 5% Columbia sheep BA and incubated at 37 C overnight and tested for antimicrobial susceptibility testing using the standard Kirby–Bauer disk diffusion method. Selection of disk concentration, test method and interpretation of zone diameter were done as recommended by the Clinical Laboratory Standards Institute (CLSI) (74, 75). *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) were used as reference strains for *E. coli* and *Enterococcus* species, respectively. The following antimicrobial agents and disk potency were used: AMX (30 µg), AMP (10 µg), apramycin (APR,15 µg), BAC (10 IU), CEF (30 µg), CHL (30 µg), CIP (5 µg), ENR (5 µg), ERY (15 µg), florfenicol (FLO, 30 µg), GEN (10 µg), GEN [(120 µg, to determine high level resistance to aminoglycosides in *Enterococcus* species)], LIN (2 µg), neomycin (NEO, 30 µg), PEN (10 units), SPE (100 µg), TET (30 µg), SXT (1.25 µg), SSS (0.25 mg) and TYL (60 µg), VAN (30 µg). The antimicrobials used in this study represented 10 classes; namely β-lactams (AMX, AMP, CEF, PEN), aminoglycosides (GEN, NEO, SPE), cyclic polypeptides (APR, BAC), phenicols (CHL, FLO), fluoroquinolones (CIP, ENR), lincosamides (LIN), macrolides (ERY, TYL), tetracyclines (TET), glycopeptides (VAN) and folate pathway inhibitors (SSS, SXT). The inhibition zone diameter of each antimicrobial agent was measured using the BIOMIC V3 -2014-Microbiology Digital Image Analysis System (Giles Scientific Inc, Santa Barbara, California, USA). Inhibition zone diameters were used to categorize antimicrobial susceptibility of the isolate as susceptible, intermediate and resistant according to the CLSI recommendations except for sulfonamides, where the European Committee on Antimicrobial Susceptibility Testing version 4.0 interpretive criteria were used (118). Multidrug resistance was enumerated as acquired non-susceptibility to at least one agent in three or more antimicrobial classes (229). Intrinsic AMR was disregarded in this enumeration.

3.4 Results

3.4.1 Antimicrobial resistance of *E. coli*

E. coli isolates were resistant to TET, AMP, AMX, SSS, CEF, GEN and SPE at the rate of 54.4%, 50.9%, 42.1%, 31.6%, 29.8%, 28.6% and 20.5% respectively. The descending order of AMR to the remainder of the antimicrobials were CIP (7.0 %), NEO (7.0 %), ENR (6.4 %), APR (5.8 %), CHL (3.5 %), FLO (3.5 %) and SXT (3.5 %) (Figure 3.1). MDR was seen in 58 of 170 (34.1 %) *E. coli* isolates of which 14.1% (n=24) of *E. coli* were resistant to three classes of

antimicrobials, 15.9 % (n=27) of *E. coli* were resistant to four classes of antimicrobials and 4.1 % (n=7) of *E. coli* were resistant to five classes of antimicrobials (Figure 3.2). The intrinsic resistance of *E. coli* was noted for BAC (99.4 %), LIN (99.4 %), TYL (98.2 %), VAN (97.7 %), PEN (97.1 %) and ERY (91.2 %). The AMR profile of all *E. coli* isolates are shown in Table 3.1 and Table 3. 2 AMR phenotypes of *E. coli,* in descending order, were TET (23/150), AMX (R) + AMP(R) + CEF(R) + GEN(R) + SPE(R) + TET(R) + SSS (R) (9/150), AMX R) + AMP (R) + CEF (R) + CIP (R) + ENR (R) + TET (R) + SSS (R) (8/150) and AMX (R) + AMP (R) + CEF (R) (8/150). Pan-resistance was not observed for *E. coli* but pan-susceptibility was observed in 21.3% isolates.

Table 3.1: AMR profile of *E. coli*

Drug class	**Drug**	**Disk potency**	**Resistance percentage** *(n=170)*
β-lactam	AMX	30 µg	42.1
	AMP	10 µg	50.9
	CEF	30 µg	29.8
Phenicols	CHL	30 µg	3.5
	FLO	30 µg	3.5
Fluoroquinolones	ENR	5 µg	6.4
	CIP	5 µg	7.0
Aminoglycosides	GEN	10 µg	28.6
	NEO	30 µg	7.0
	SPE	100 µg	20.5
Tetracyclines	TET	30 µg	54.4
Cyclic polypeptides	APR	15 µg	5.8
Folate pathways inhibitors	SSS	31.58 µg	31.6
	SXT	1.25/23.75 µg	3.5

Table 3.2: Summary of AMR of *E. coli* (n=170)

Resistance profile										Isolates (n=)
AMX	AMP	CEF	CIP	ENR	GEN	SPE	TET	SXT	SSS	2
AMX	AMP	CEF	CHO	FLO	GEN	NEO	TET	SSS		1
AMX	AMP	CEF	CHO	FLO	GEN	SPE	TET	SSS		1
AMX	AMP	CEF	CIP	GEN	SPE	TET	SSS			1
AMX	AMP	CHO	FLO	GEN	SPE	TET	SSS			1
AMX	CEF	CHO	FLO	GEN	SPE	TET	SSS			1
AMX	AMP	CEF	GEN	SPE	TET	SSS				9
AMX	AMP	CEF	CIP	ENR	TET	SSS				8
AMX	AMP	CEF	CHO	FLO	TET	SSS				1
AMX	AMP	GEN	NEO	TET	SXT	SSS				1
AMX	AMP	APR	CEF	GEN	NEO	TET				1
AMX	AMP	CEF	GEN	SPE	SSS					3
AMX	AMP	GEN	SPE	TET	SSS					3
AMX	AMP	CEF	GEN	TET	SSS					1
AMX	AMP	CEF	CIP	ENR	SSS					1
AMX	AMP	CEF	FLO	TET	SSS					1
AMX	AMP	APR	CEF	GEN	NEO					1
AMX	AMP	APR	CEF	NEO						2
AMX	AMP	CEF	GEN	SSS						1
AMX	AMP	GEN	NEO	TET						1
AMX	AMP	GEN	TET	SSS						1
AMX	AMP	TET	SXT	SSS						1
AMX	AMP	CEF	TET							4
AMX	AMP	CEF	GEN							1
AMX	AMP	CEF	SPE							1
AMX	AMP	GEN	TET							2
AMP	GEN	SPE	TET							2
AMX	AMP	SPE	SSS							1
AMX	AMP	TET	SSS							1
AMX	SPE	TET	SSS							1
APR	GEN	NEO	SPE							1
GEN	SPE	TET	SSS							5
GEN	SPE	TET	SXT							1
AUG	AMP	CEF								8
AMX	AMP	TET								5
GEN	SPE	SSS								3
AMP	GEN	TET								2
NEO	TET	SSS								2
AMP	CEF	GEN								1
AMP	SPE	SSS								1
AMP	SXT	SSS								1
APR	NEO	TET								1
GEN	TET	SSS								1
AMP	TET									5
AUG	AMP									2
AMP	GEN									2
AMP	APR									1
APR	NEO									1
TET										23
AMP										3
APR										1
Pan-susceptible										32
Other (non-characterized)										12

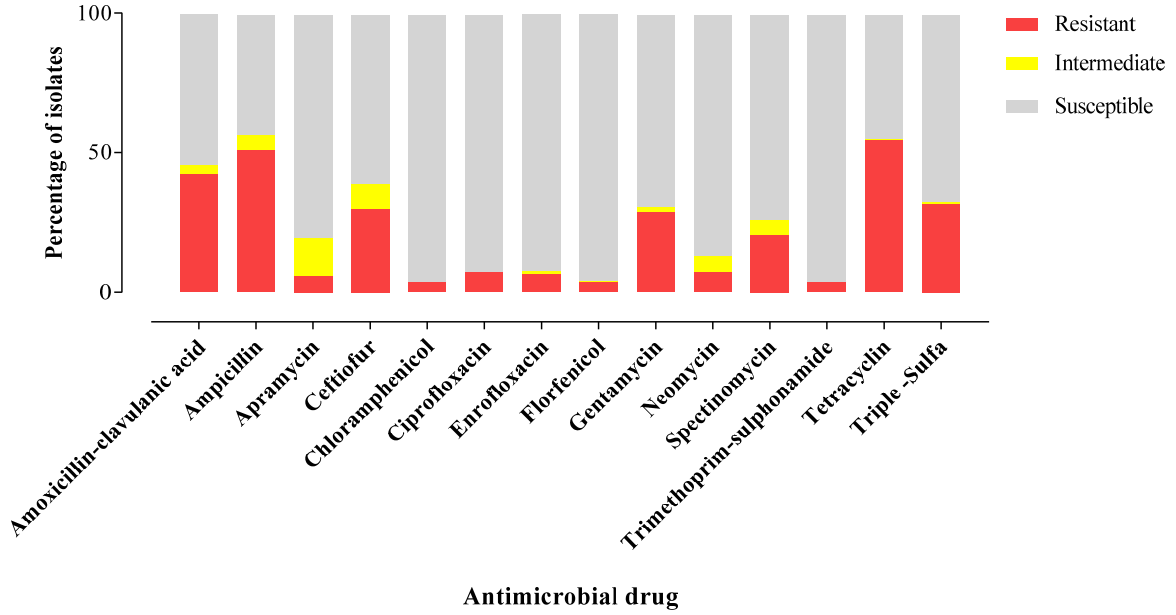

Figure 3.1: AMR profile of *E. coli*

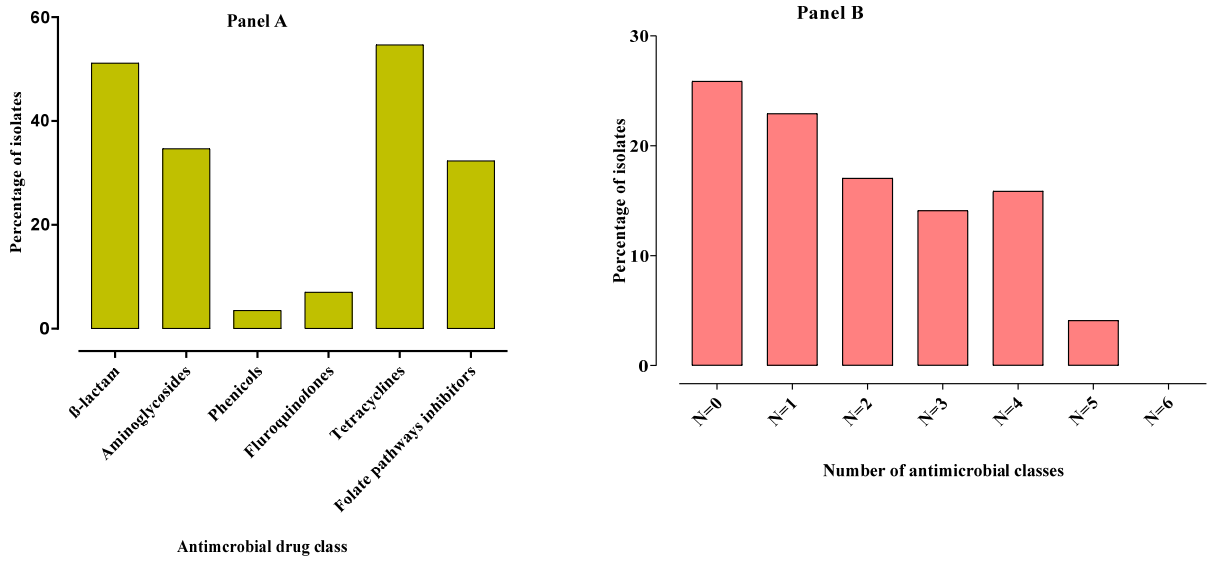

Figure 3.2: (Panel A) AMR profile of *E. coli* to each drug class and (Panel B) indicates the MDR profile of *E. coli*

3.4.2 Antimicrobial resistance of *Enterococcus* species

All *Enterococcus* isolates were resistant to at least one antimicrobial agent. AMR phenotypes of *Enterococcus* isolates, in descending order, were TET (73.4%), CEF (51.9%), BAC (42.6%), ERY (31.2%), TYL (30.1), NEO (27.7%), GEN (8.98%), SPE (8.98%), PEN (7.8%), SXT (7.4%), ENR (5.1%), AMP (2.7%), CHL (2.7%), VAN (1.9%), CIP (1.6%), AMX (0.4%) and FLO (0.4%) (Figure 3.3). Only 3.9 % (10/256) of *Enterococcus* isolates were resistant to high concentration of GEN. MDR was seen in 44.9% *Enterococcus* isolates of which 25.8%, 14.4%, 2.3%, 0.8% and 1.6% of *Enterococcus* isolates were resistant to three, four, five, six, and seven classes of antimicrobials, respectively (Figure 3.4). No pan-resistant or pan-susceptible *Enterococcus* isolates were observed. The intrinsic resistance of *Enterococcus* isolates were noted for APR (98.83%) and LIN (96.88%).

AMR profiles of *E. faecalis* and *E. faecium* were summarized in Table 3.3. The descending order of AMR of *E. faecalis* were; TET (73.1%), CEF (47.98%), BAC (43.9%), ERY (31.4%), TYL (30.5%), NEO (26.9%), GEN (9.9%), SPE (6.3%), CHL (3.1%), SXT (1.8%), VAN (1.8%), PEN (1.3%), ENR (1.3%), AMP (0.4%), CIP (0.4%), AMX (0.4%) and FLO (0.4%) (Figure 3.5). Only 6.3% (14/223) of *E. faecalis* isolates were resistant to high concentration of GEN. Multidrug drug resistance was seen in 35.9% of *E. faecalis* isolates of which 26.9% of *E. faecalis* isolates were resistant to three classes of antimicrobials, 11.7% of *E. faecalis* isolates were resistant to four classes of antimicrobials, 1.8% of *E. faecalis isolates were* resistant to five classes of antimicrobials and 0.9% of *E. faecalis* isolates were resistant to six classes of antimicrobials (Figure 3.5). The resistance profiles of all *E. faecalis* isolates are demonstrated in Table 3.4. The most common resistance phenotype of *E. faecalis* was TET (R) + BAC (R) (37/223) followed by TET (R) + CEF (R) (23/223), TET (12/223) and TET (R) + ERY (R) + NEO (R) + TYL (R) (12/223).The descending order of resistance of *E. faecium* was CEF (95.2%), PEN (80.9%), SXT (66.7%), TET (61.9%), NEO (47.6%), BAC (42.9%), ENR (42.9%), ERY (38.1%), TYL (38.1), SPE (33.3%), AMP (28.6%), CIP (14.3%) and GEN (4.8%). No *E. faecium* was found resistant to AMX, CHL, FLO and VAN (Figure 3.6). MDR was seen in 85.7% of *E. faecium* isolates of which 19.0% of *E. faecium* were resistant to three classes of antimicrobials, 38.1% of *E. faecium* were resistant to four classes of antimicrobials, 9.5% of *E. faecium* were resistant to five classes of antimicrobials and 19.0% of *E. faecium* were resistant to seven classes of antimicrobials (Figure

3.6). The resistance profiles of all *E. faecium* isolates were shown in Table 5. The most common resistance phenotype was CEF (R) + NEO (R) + TET (R) + SXT (R) + PEN (R) (4/21).

Table 3.3: AMR profile of *E. faecalis and E. faecium*

Drug class	Drug	Disk potency	Resistance percentage	
			***E. faecalis* (n=223)**	***E. faecium* (n=21)**
β-lactam	AMX	30 µg	0.4	0
	AMP	10 µg	0.4	28.6
	PEN	10 IU	1.3	80.9
	CEF	30 µg	47.98	95.2
Phenicols	CHL	30 µg	3.1	0
	FLO	30 µg	0.4	0
Fluoroquinolones	ENR	5 µg	1.3	42.9
	CIP	5 µg	0.4	14.3
Macrolides	ERY	15 µg	31.4	38.1
	TYL	60 µg	30.5	38.1
Aminoglycosides	GEN	10 µg	9.9	4.8
	NEO	30 µg	26.9	47.6
	SPE	100 µg	6.3	33.3
Tetracycline	TET	30 µg	73.1	66.7
Folate pathways inhibitors	SXT	1.25/23.75 µg	1.8	61.9
Glycopeptides	VAN	30 µg	1.8	0

Table 3.4: Summary of AMR profiles of *E. faecalis* (n=223)

Resistance Profiles							Isolates (n=)
TET	BAC	CEF	ERY	TYL	NEO		1
TET	BAC	CEF	ERY	TYL			2
TET	BAC	CEF	NEO	GEN			1
TET	BAC	CEF	GEN				2
TET	BAC	CEF					6
TET	BAC	GEN					1
TET	BAC	ERY	TYL	GEN			1
TET	BAC	ERY	TYL	NEO			1
TET	BAC	ERY	TYL				6
TET	BAC						37
TET	CEF	ERY	NEO	TYL			8
TET	CEF	ERY	NEO				1
TET	CEF	GEN					5
TET	CEF	NEO					6
TET	CEF						23
TET							12
TET	ERY	TYL					7
TET	ERY						1
TET	ERY	GEN	NEO	TYL			1
TET	ERY	NEO	TYL				12
TET	GEN						8
TET	NEO						6
BAC	CEF	ERY	NEO				2
BAC	CEF	ERY	GEN				1
BAC	CEF	ERY					4
BAC	CEF	NEO					2
BAC	CEF						5
BAC	ERY	NEO	TYL				1
BAC							2
CEF	ERY	TYL	NEO				2
CEF	ERY	TYL					6
CEF	NEO	GEN					1
CEF	NEO						3
CEF							5
ERY	TYL						2
ERY	TYL	NEO					1
AMX	GEN	TET					1
AMP	CEF	ENR	PEN				1
CHO	BAC	ERY	TET	TYL			4
CHO	BAC	ERY	ENR	TET	TYL	NEO	1
CHO	BAC	ERY	ENR	TET	TYL		2
CIP	CEF	ENR	PEN				1
FLO	CEF	GEN	TET	SXT	VAN		1
PEN	CEF	BAC	TET				1
PEN	CEF	CIP	ENR				1
SPEC	BAC	NEO	TYL	ERY	NEO		1

SPEC	BAC	CEF	NEO			5
SPEC	BAC	CEF				4
SPEC	BAC	NEO				1
SPEC	BAC					3
SXT	TET	NEO				1
SXT	TET	NEO	CEF			1
VAN	TYL	TET	NEO	ERY	CEF	1
VAN	BAC	ERY	TET	TYL		1
VAN	CEF					1
Other (non-characterized)						6

Table 3.5: Summary of AMR of *E. faecium* (n=21)

Resistance profile												Isolates (n=)
CEF	NEO	TET	SXT	PEN								4
AMP	CEF	ENR	PEN	SXT	BAC	ERY	NEO	TET	SPEC	TYL		2
AMP	CEF	ENR	PEN	SXT								2
CIP	AMP	BAC	CEF	ENR	ERY	NEO	PEN	SPEC	TET	SXT	TYL	1
CIP	ENR	GEN	SXT									1
CIP	AMP	CEF	ENR	ERY	PEN	SXT						1
BAC	CEF	TET	PEN	ENR	ERY	NEO	SPEC	SXT	TYL			1
BAC	CEF	TET	PEN	SPEC								1
BAC	CEF	TET	PEN	ERY	NEO	TYL						1
BAC	CEF	TET	PEN	SPEC								1
BAC	CEF	TET	PEN	ENR	ERY	TYL						1
BAC	CEF											1
CEF	PEN	SXT										1
CEF	SPEC	TYL										1
CEF	ERY	PEN	TYL									1
CEF	NEO	TET	SXT	PEN								1

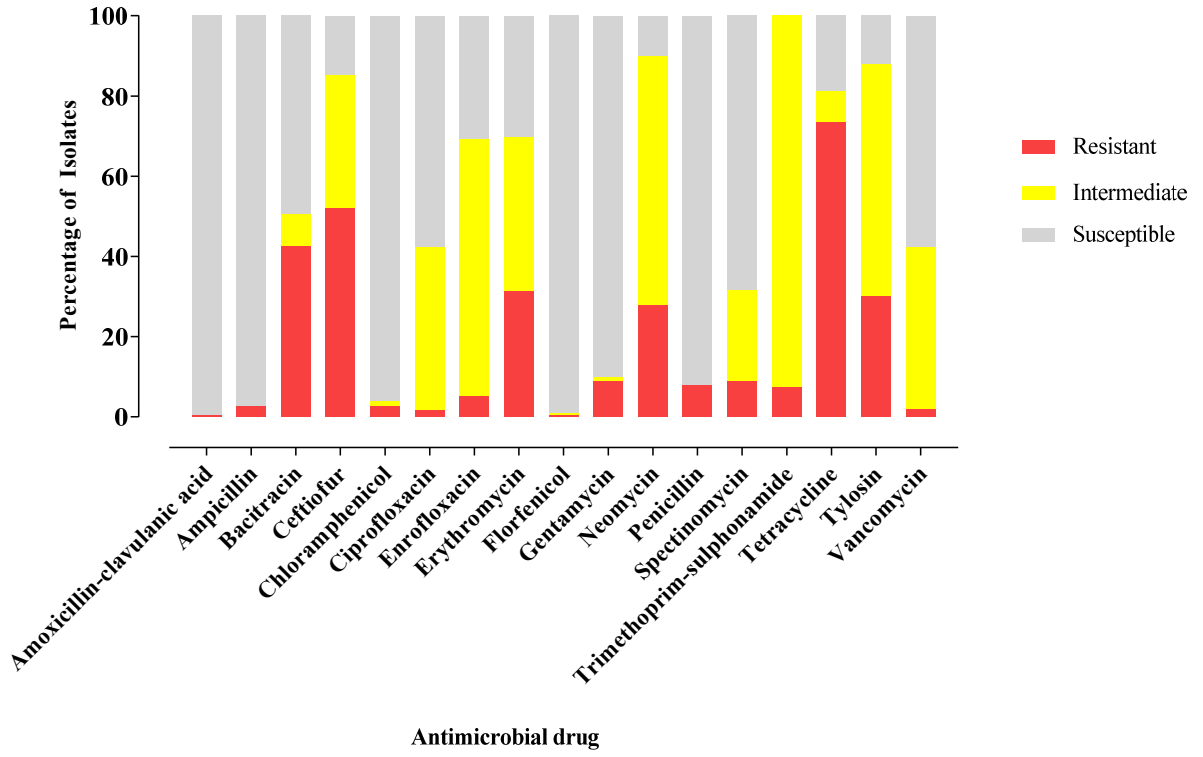

Figure 3.3: AMR profiles of *Enterococcus* species. The descending order of resistance was seen for TET, CEF, BAC, ERY and TYL**.**

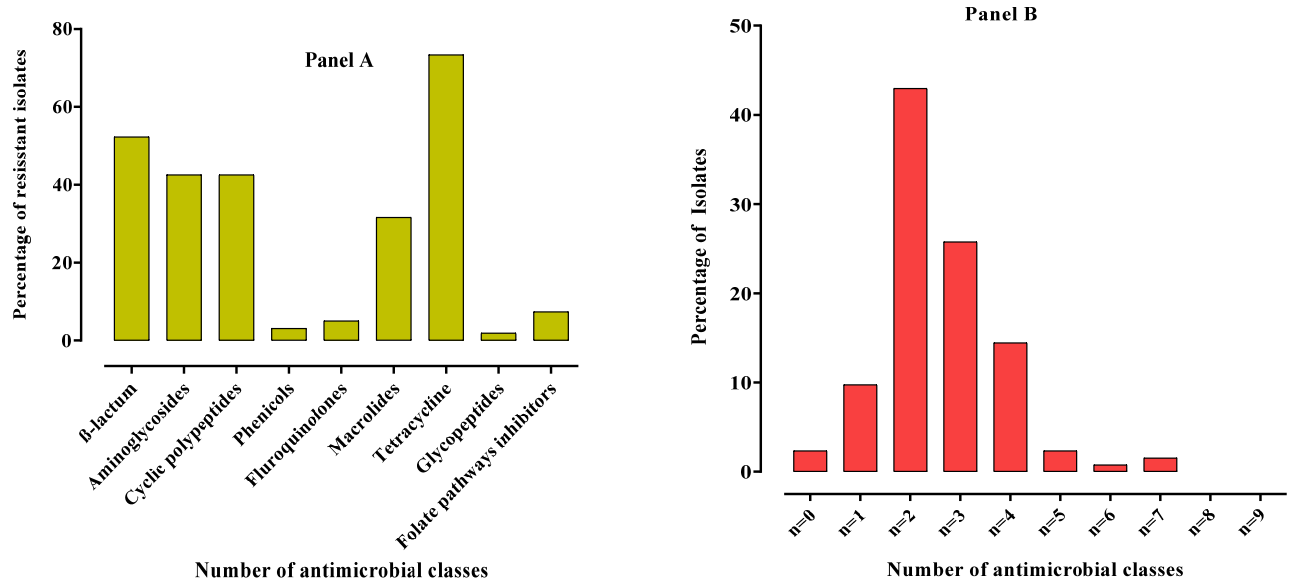

Figure 3.4: AMR profiles of *Enterococcus* species to different classes of antimicrobials (Panel A) and MDR profiles of *Enterococcus* species (Panel B)

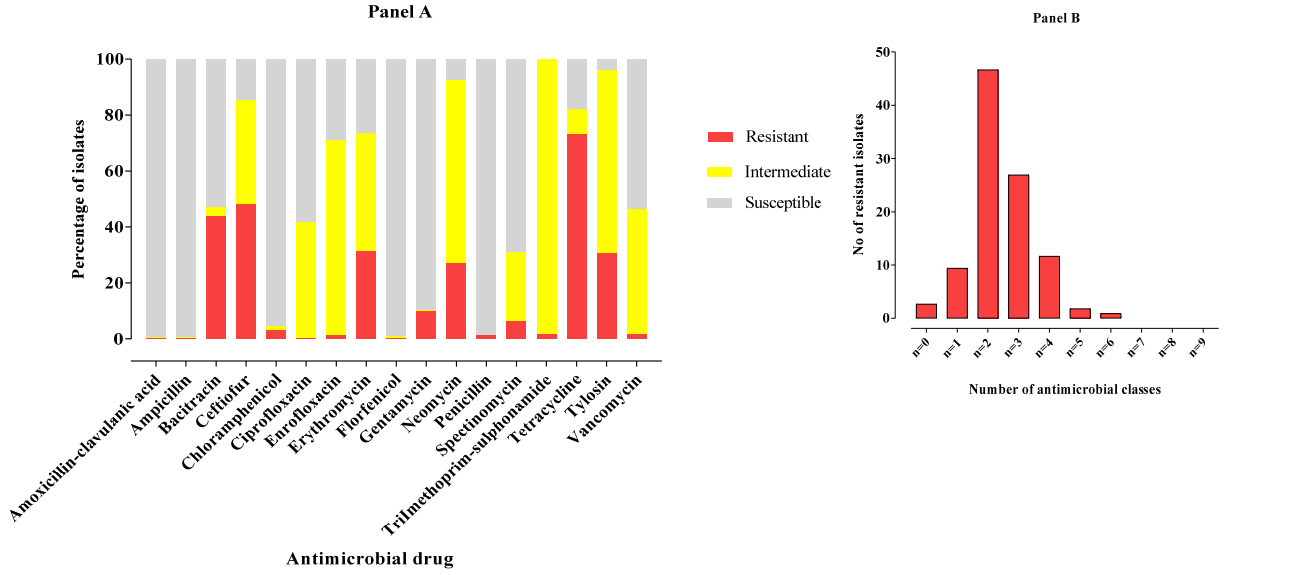

Figure 3.5: AMR profiles of *E. faecalis* (Panel A) and indicates MDR profiles of *E. faecalis* (Panel B)

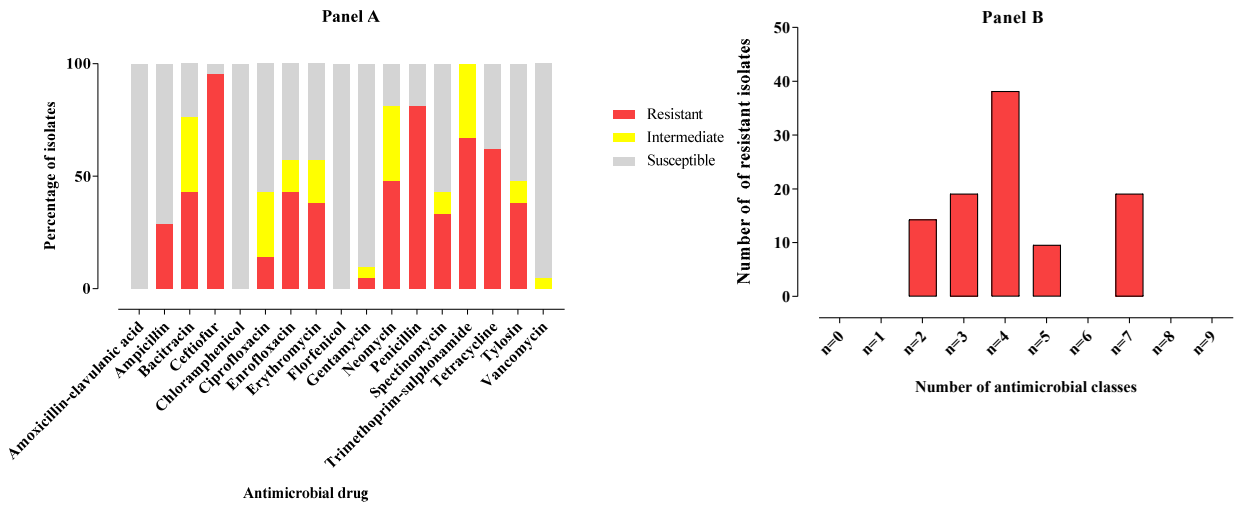

Figure 3.6: AMR profiles of *E. faecium* (Panel A) and indicates MDR profiles of *E. faecium* (Panel B).

3.5 Discussion

The emergence of AMR is a serious threat to global health, and thus the WHO has recently declared a priority list of pathogens which need novel antibiotic development (276). Multidrug resistance is a worldwide concern due to failures in treating infectious diseases. The resistance genes are often on mobile genetic elements, including plasmids, integrons, and transposons (131). The resistance genes are transferred among bacteria via horizontal gene transfer, conjugation, transformation and transduction, which ultimately encodes for multidrug resistance (182). The present study was designed to investigate the AMR profiles of *E. coli* and *Enterococcus* species isolated from non-viable chicken embryos, an overlooked niche concerning the emergence of multidrug-resistant bacteria.

Our data showed a high degree of resistance of *E. coli* to β-lactam antimicrobials; AMP (42.1%) and AMX (50.9%). Our data in regards to AMP resistance is comparable with AMP resistance of *E. coli* isolated (43%) from poultry products in Canada by the CIPARS in 2016 (72). A recent study has described the emergence of extended-spectrum β-lactamases (ESBLs)-encoding plasmids from *E. coli* isolates in poultry with a similar rate of prevalence as observed in humans which warrants regular monitoring of AMR in the broiler industry (390). We observed a relatively high prevalence of CEF resistance in *E. coli* (29.8%) which justifies the voluntary withdrawal of this antimicrobial from poultry production in 2014 (72). It would be interesting to

study CEF resistance in *E. coli* from chicken embryo mortality in a few years, since CEF resistance of *E. coli* in poultry hatcheries may impose a risk of dissemination to humans. It has been reported that *E. coli* of poultry origin are closely related to *E. coli*-associated extra-intestinal infections in humans (35). When compared to GEN resistance reported by CIPARS in poultry products (9%), the prevalence was higher in *E. coli* isolated from dead embryos (72). CIPARS data represent the overall Canadian poultry industry, which may overlook this emerging ecological milieu in western Canada. However, both CARSS and CIPARS have documented an increased trend in GEN resistance in *E. coli* isolates of poultry origin from 2004-2014 (59). GEN is used in the poultry industry to reduce neonatal poultry mortality and for growth promotion (236). Hence, we can speculate an association between GEN use and increased *E. coli* resistance to GEN in the poultry industry in western Canada. In our study, 54.4 % of *E.coli* were TET resistant, which is comparable with CIPARS data as they have observed 50 % of *E. coli* resistant to TET in 2016 (72). This trend may be explained by the heavy use of TET in the poultry industry in Canada (105, 107). At present, there are 38 different TET resistance genes described (304), and further investigation is needed to characterize these genes in isolates recovered in our study to determine the resistance mechanisms.

We have seen 1.9% VRE in dead chicken embryos although VAN has not been used in the broiler chicken industry in Canada. The mean VRE incidence increased from 6.2% in 2011 to 7.9% in 2014 in Europe. The frequency of VRE ranged from 0% (Estonia, Finland, Iceland, and Malta) to 45.1% (Ireland). In 2014, increasing trends of VRE were seen in Bulgaria, Croatia, Denmark, Hungary, Ireland, Italy, Slovakia, and United Kingdom from 2011 to 2014 (86). A study conducted in British Columbia, Canada in 2010 investigating *Enterococcus* isolates obtained from fecal and cecal contents of commercial poultry, demonstrated that none of the enterococci were resistant to VAN (106). Enterococci of foodborne origin were not identified as a direct cause of resistant Enterococci in humans, but they could pose a risk in transfer of resistance determinants to human-adapted strains of the same genus or other genera, as shown in VAN resistance in *S. aureus* and TET and ERY resistance in *Listeria monocytogenes* (23, 159, 169).

The resistance of enterococci to TET (73.4%), BAC (42.6%) and TYL (30.1%) was remarkable in our study. It has also been suggested that commensal microbiota of poultry can be a reservoir of BAC resistance, and this BAC resistance can be readily transferable to *E. faecalis* in humans (67). Genes encoding resistance to TET, *tetL* and *tetM,* are frequently associated with *ermB* which encodes resistance to macrolides, lincosamides, streptograminB and quinupristin-

dalfopristin. Since BAC is commonly used as a growth-promoting antibiotic in the Canadian poultry industry, resistance to BAC and other antibiotics mentioned above can be co-selected (67). A recent study conducted in Asia looked at determining AMR of uropathogenic *E. coli* and APEC and found MDR in 98% of isolates where most of them were resistant to at least five antimicrobials tested (197). Moreover, emerging ESBLs producing *E. coli* were resistant to aminoglycosides and fluoroquinolones (306). Among them, a classic example of globally disseminated, MDR *E. coli* strain sequence type 131 (ST131) which causes significant amounts of the UTI and bloodstream infections in humans (266, 288).

In the present study, we have observed that chicken embryos harbor a significant number of multidrug-resistant *E. coli* and enterococci species, revealing that this niche can be a substantial source of superbugs in the environment. The current AMR surveillance systems predominantly focus on monitoring resistance in poultry farms and processing plants. Embryonated eggs represent a critical niche that can reveal the nature of AMR that would be passed on to the production farms and ultimately to humans via poultry products. Our data suggest that screening of AMR, particularly at the level of embryonated eggs, is essential in AMR surveillance to understand AMR dissemination for developing appropriate control measures.

PREFACE TO CHAPTER 4

We have demonstrated in Chapter 2 that the majority of non-viable chicken embryos were positive for bacterial growth. It was found that 68.52% of non-viable chicken embryos were positive for at least one type of bacterial species. Furthermore, we have demonstrated in Chapter 2, more than 50% of *Enterococcus* infected embryos were co-infected with *E. coli*. Enterococci and *E. coli* were the most common bacterial species recovered among them. Moreover, the occurrence of MDR is prevailing in these isolates as we have demonstrated in Chapter 3. Transmission of bacteria through the eggshell is widely studied. Once bacteria overcome natural defense barriers of eggshell and shell membranes, they can enter the interior of the developing embryo (134). Experimental studies have demonstrated that contamination of embryonating eggs with *E. coli*, *E. cecorum* and *E. faecalis* can result in embryo mortality during incubation. Although, most of the dead embryos were positive for enterococci and *E. coli,* host-pathogen interactions of enterococci and *E. coli* are not clearly understood. Hence, in Chapter 4, we will study the migration of *E. faecalis* and *E. coli* in internal organs following incubating eggs which were dipped in a solution containing bacteria and cytokine responses of SPF chicken embryos and neonatal chickens.

Chapter 4 SYNERGISM OF *E. FAECALIS* AND *E. COLI* IN CHICKEN EMBRYOS RESULTS IN INCREASED NEONATAL CHICKEN MORTALITY

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The manuscript is in preparation for publication.

Contribution: The experiments are designed by my self and Dr. Gomis. The members of the research group assisted to conduct the animal experiments and laboratory procedures. I wrote the manuscript under Dr. Gomis' guidance and all the authors mentioned above contributed with their feedback.

4.1 Abstract

Enterococci and *E. coli* are opportunistic pathogens of poultry and associated with embryo and neonatal chick mortality. Recently we have reported a possibility of increased neonatal broiler chicken mortality following coinfection of chicken embryos with enterococci and *E. coli* in poultry hatcheries, a finding not demonstrated experimentally in chickens previously. The objective of this study was to investigate the host pathogen interactions of *E. faecalis* and *E. coli* in developing SPF chicken embryos and neonatal chickens. Hatching eggs at day-12 of incubation were exposed to *E. faecalis* and/or *E. coli* on the eggshell by dipping eggs for 30 sec in a solution containing different doses of *E. faecalis* and/or *E. coli* in order to study the migration of *E. faecalis* and *E. coli* during egg incubation and following hatch. A MDR isolate of *E. faecalis* was able to colonize multiple internal organs of chicken embryos quickly compared to an *E. faecalis* isolate from a healthy chicken without affecting viability or hatchability of embryos. Although *E. faecalis* was colonized internal organs of chicken embryos, no histopathological lesions of inflammation or expression of virulence genes of *E. faecalis* was observed. Moreover, no expression of host cytokine/chemokine genes during *E. faecalis* colonization in embryos was seen, except there was a trend in host cytokine response against *E. faecalis* and *E. coli* at day-20 of incubation. Although, no mortality of embryos was observed during incubation in groups exposed to both *E. faecalis* and *E. coli,* neonatal chicken mortality reached 13.3%. These findings suggest that *E. faecalis* may down regulate or evade the host immune system of developing chicken embryos and vertically transfer *E. faecalis* in neonatal chickens. Furthermore, synergistic interactions of *E. faecalis* and *E. coli* in chicken embryos lead to increased mortality in neonatal chickens. This study highlights the complex nature and differences of interactions of *E. faecalis* in chicken embryos and neonatal chickens and resulting pathology and mortality of chickens.

4.2 Introduction

Enterococci are opportunistic pathogens and cause nosocomial infections, such as bacteremia, septicemia, valvular endocarditis, UTI, intra-abdominal, and pelvic infections in humans (249). Among different species of enterococci, *E. faecalis* and *E. faecium* are responsible for the majority of infections (269). Therapeutic failures associated with MDR enterococci such as VRE cause higher mortality among immunocompromised and debilitated patients (22, 178, 269). A variety of invertebrate and vertebrate models of enterococci infections have been developed to study pathogenic mechanisms, virulence determinants relating to adherence, colonization, internalization, extracellular survival and immune evasion, however, the pathogenesis of enterococci associated infections are poorly understood (136, 144).

Enterococcus infections of poultry have become an emerging problem in the recent past and cause significant economic losses to the industry worldwide. Among those, *E. cecorum* associated with osteomyelitis and spondylitis in broilers and broiler breeders and *E. faecalis* associated with amyloid arthropathy in table-egg layers were the primary economically important pathogens to the poultry industry (186, 214). *E. hirae* and *E. durans* have been associated with encephalomalacia and endocarditis in broiler breeders and young chicks with high mortality (3). Although researchers have been successful in reproducing *Enterococcus* infections in chickens (63, 211, 237), the reasons for the emergence of *Enterococcus* infections in the poultry industry is not clear. Bacterial colonization in the intestine of chicken embryos starts early during embryo development and the composition of bacterial species changes during egg incubation (108). Based on a whole genome sequence analysis study, it was found that bacterial species belong to 162 genera were present in broiler breeders and their eggs during embryo development. Of those 162 genera, 65 genera including the genus *Enterococcus* were found during the entire duration of embryo development (108). Bacteria belonging to the genus *Enterococcus* are found in low numbers in embryos during the last days of incubation while they are predominant in newly hatched neonatal chicks (108, 126). The chicken intestinal tract is colonized with *E. faecalis*, *E. faecium*, *E. hirae*, *E. durans*, *E. casseliflavus, E. gallinarum* and *E. avium* demonstrating that *enterococcus* are one of the most predominant genera of bacteria in the chicken intestinal flora (100, 326). *E. faecalis* are the most abundant inhabitants of chicken intestines among other species of enterococci in the first 3 weeks of a chick's life. It has been reported that colonization of *E. faecalis* occur in neonatal chickens likely due to cloacal drinking following hatch (100, 127). Once

the intestinal barrier function is interrupted due to either immunosuppression or abdominal infections, enterococci can penetrate the intestinal mucosa and initiate systemic infections (227, 238, 365).

APEC causes extra intestinal tract infections in neonatal broiler chickens, predominantly YSI, omphalitis, respiratory infections, swollen head syndrome, septicemia and cellulitis. *E. coli* causes high mortality in broilers following hatch and acute septicemia and subsequent subacute to chronic conditions such as pericarditis, airsacculitis, perihepatitis, arthritis and osteomyelitis during grow out period. Bacteremia appears to be essential in the development of *E. coli* septicemia but the pathogenies is poorly understood (148). *E. coli* has been isolated concurrently with enterococci from chicken embryos and neonatal chickens (23). *Enterococcus* infections together with *E. coli* infections in broiler chicken, leading to YSI and have been emerging recently (193).

It has been reported that polymicrobial infections result in synergism of microorganisms and disease in the host (167). Among polymicrobial infections, catheter associated UTI in humans were reported commonly in hospitals. Studies of wound infections in a mouse model suggest that *E. faecalis* promotes *E. coli* biofilm formation under low-iron availability thus facilitating polymicrobial infections. It has also been reported in catheter associated UTI in a mouse model, that synergism of enterococci and *E. coli* resulted in suppression of NF-κB pathways of macrophages and promote polymicrobial infections (167, 358).

Chicken embryo mortality and YSI in neonatal chickens due to enterococci and *E. coli* are economically important since these bacterial infections lead to chronic infections and high mortality, poor growth and performance and increased condemnations at processing (177, 298). Various infectious and non-infectious causes have been associated with chicken embryo mortality, among them bacterial infections were recognized as the main cause of embryo mortality (9, 26, 191). It has been demonstrated that fecal contamination of egg shells and vertical transmission of bacteria in hatching eggs resulted in embryo mortality and poor hatchability (37) (79). In the recent past, the incidence of *Enterococcus*-associated YSI has been increasing in the poultry industry worldwide, including in Canada. Historically, *E. coli* associated YSI accounted for the majority of embryo deaths compared to *Enterococcus* species (26). Recently, we have reported that a majority of unhatched broiler embryos at 21 days of incubation were positive for mainly *Enterococcus* species followed by *E. coli*. We have also found that 56% of dead embryos had coinfection of *Enterococcus* species with *E. coli*. Among *Enterococcus* species, *E. faecalis* was the predominant

Enterococcus species followed by *E. faecium* (193). Although, *E. faecalis* and *E. coli* infections of broiler chicken embryos and neonatal broiler chickens are common, the synergistic effects of *E. faecalis* with *E. coli* are poorly understood in chickens. Therefore, the objective of this study was to investigate the host pathogen interactions of *E. faecalis* and *E. coli* in developing chicken embryos and during the neonatal period.

4.3 Materials and methods

4.3.1 Bacterial isolates and growth conditions

4.3.1.1 Selection of *Enterococcus* isolates

Three *E. faecalis* isolates were used in the experiments. First, an *E. faecalis* isolate was recovered from the rectum of a healthy broiler breeder chicken at 32 weeks of age and was used as the control strain. The second *E. faecalis* isolate was obtained from the yolk sac of an early dead embryo from a broiler hatchery (193) and was determined to MDR (resistant to BAC, CEF, ERY, LIN, NEO, TET, SSS and TYL. Finally, the third *E. faecalis* isolate was obtained from the YS of a 3-day-old neonatal broiler chicken that died of YSI. All three isolates were stored in BHI broth (Becton, Dickinson & Company, France) supplemented with 20% glycerol (w/v) (Fisher Scientific, Fair lawn, New Jersey) and stored at -80 C prior to animal experiments.

4.3.1.2 Selection of the *E. coli* isolate

A field strain of *E. coli* from a turkey with septicemia was also used in the second experiment in addition to *E. faecalis* MDR isolate. This *E. coli* strain was serogroup O2, non-hemolytic, serum-resistant, produced aerobactin, with a K1 capsule and Type 1 pili (153). A single colony of *E. coli* was added to 100 mL of Luria Bertani (LB) broth (Difco LB broth Miller; Becton, Dickinson, and Company, Sparks, MD) in a 250 mL Erlenmeyer flask. The culture was grown at 37 C for 12 hr, shaking at 150 rpm. This stationary phase culture contained approximately $1x10^9$ CFU/mL bacteria. Then, 10 mL of bacterial broth was transferred to three flasks each containing 1L of Luria broth. Bacteria inoculated LB was then incubated at 37 C in a shaking incubator for 12-13 hr to obtain $1x10^9$ CFU/mL. Then dilutions were then made to obtain $1x10^6$ CFU/mL and $1x10^3$ CFU/mL bacteria.

4.3.1.3 Preparation of *E. faecalis* and *E. coli* for experimental challenge

Each isolate of *E. faecalis* was streaked on 5% Columbia sheep BA (Oxoid, Nepean, Ontario, Canada) and incubated aerobically at 37 C for 24 hr. A single colony was picked and inoculated in 100 mL Todd Hewitt broth and incubated at 37 C for 12-13 hr on a shaker. According to growth curve analysis, each *E. faecalis* strain reached $1x10^9$ CFU /mL at the end of the incubation period. Following incubation a 1:100 dilution was made in Todd Hewitt broth and incubated at 37 C for 4 hr on a shaker in order to bring *E. faecalis* to log phase at a concentration of $1x10^9$ CFU/mL.

For *E. faecalis* and *E. coli* coinfection experiment, *E. faecalis* $1x10^9$ CFU/mL concentration in 1.5 L of Todd Hewitt with corresponding *E. coli* concentrations in 1.5 litres were prepared and mixed in 6 L plastic containers and allowed to cool down to 10 C.

Viable bacterial counts of *E. faecalis* or *E. coli* were determined by plating serial dilutions of bacterial cultures in duplicate before and after infection in either m-Enterococcus agar or MacConkey agar to enumerate *E. faecalis* and *E. coli*, respectively.

4.3.2 Experimental design

4.3.2.1 (Experiment A): *E. faecalis* associated pathology and cytokine response of chicken embryos

The objectives of this study were to explore the migration of enterococci from the eggshell to internal organs, histopathological and electron microscopical changes of chicken embryos, expression of virulence factors of enterococci and expression of host cytokines during the course of infection. An *E. faecalis* model was used as previously described with a few modifications (23). Briefly, a temperature gradient was maintained between SPF incubating eggs at 12 days of incubation (37 C) and bacterial broth (10 C) to facilitate the entrance of *E. faecalis* into incubating eggs. Eggs were candled to determine embryo viability prior to *E. faecalis* infection. Viable eggs were divided into 6 groups (n=60). The first group was maintained as non-dip group. The second group was dipped in sterile saline and the third group was dipped in Todd Hewitt media broth. The remaining three groups were dipped in *E. faecalis* solution individually. (*E. faecalis* isolates were recovered from a healthy chicken, case of embryo death associated with MDR and a case of YSI as described above) Three liters of bacterial broth containing $1x10^9$ CFU/mL were prepared for each *E. faecalis* isolate and maintained at 10 C during the experiment. Eggs from each group were

immersed in the respective bacterial solution for 30 sec and held at room temperature to air dry. All eggs were placed in incubators until hatch. Bacterial swabs were taken from the outer shell, inner shell, shell membrane, amnion at 6 and 8-d post-infection while bacterial swabs were collected from the intestine, liver, lung, yolk, and brain at 48 hr, 6 and 8-d post-infection from 5 viable embryos per group and any dead embryos at any time point. Presence of bacteria and bacterial counts were obtained from direct cultures and enriched cultures. Samples from intestine and liver were collected from 3 live embryos from each time point for total RNA extraction followed by host cytokine and chemokine expression level determination. The expression levels of putative virulence genes of *E. faecalis* after embryo infection were studied using intestine, liver, YS and brain samples collected at 48 hr, 6 and 8-d post-infection. YS, liver and lung samples were collected at 6 and 8-dpost-infection for histopathology. YS samples were collected from five live embryos at 6 post-infection for electron microscopic observation.

4.3.2.2 (Experiment B): *E. faecalis* together with *E. coli* associated pathology in chicken embryos and neonatal chickens

The objectives of this study were to explore the colonization of *E. faecalis* and *E. coli* in the yolk, and histopathology and mortality of neonatal chickens. A MDR *E. faecalis* isolate obtained from the YS of an early dead embryo and the *E. coli* isolate mentioned above was used for this experiment. Viable SPF eggs at day 12 of incubation were divided into 9 groups (n= 60) as (1) non-dip; (2) Todd Hewitt broth dip; (3) *E. faecalis* alone ($1x10^9$ CFU/mL); (4) *E. coli* ($1x10^9$ CFU/mL); (5) *E. coli* ($1x10^6$ CFU/mL); (6) *E. coli* ($1x10^3$ CFU/mL); (7) *E. faecalis* and *E. coli* ($1x10^9$ and $1x10^3$ CFU/mL); (8) *E. faecalis* and *E. coli* ($1x10^9$ and $1x10^6$ CFU/mL); and (9) *E. faecalis* and *E. coli* ($1x10^9$ and $1x10^9$ CFU/mL). Incubating SPF eggs were exposed to *E. faecalis* and *E. coli* as described in experiment A. Following hatch neonatal chicks from five groups [non dip, *E. faecalis* alone ($1x10^9$ CFU/mL), *E. coli* alone ($1x10^9$ CFU/mL), *E. faecalis* together with *E. coli* ($1x10^9$ and $1x10^3$ CFU/mL) and *E. faecalis* together with *E. coli* ($1x10^9$ and $^{1x}10^6$ CFU/mL)] (n=15/group) were monitored for clinical signs and mortality for one week following hatch. Bacterial swabs were taken from the yolk at 48 hrs, 6 and 8-d of post-infection from 5 viable embryos per group and any dead embryos at each time point. YS, liver, lung and heart from any dead chicks following hatch were collected for histopathology.

4.3.2.3 (Experiment C): *E. faecalis* together with *E. coli* associated cytokine response in embryos and neonatal chickens

The objectives of this study were to explore host cytokine response of chicken embryos and neonatal chickens, and neonatal chicken mortality following exposure of *E. faecalis* and *E. coli* to chicken embryos. The MDR *E. faecalis* isolate and *E. coli* used in this experiment are mentioned above. Viable SPF eggs were exposed to *E. faecalis* and *E. coli* as in experiment A described above, at day-12 of incubation (n= 100) as (1) non-dip; (2) *E. faecalis* alone ($1x10^9$ CFU/mL); (3) *E. coli* alone ($1x10^6$ CFU/mL) and (4) *E. faecalis* and *E. coli* ($1x10^9$ and $1x10^6$ CFU/mL) respectively. Following hatch neonatal chicks (n=40 /group) were kept to monitor clinical signs and mortality for two week. The lungs and intestines were collected from 3 live embryos and 3 live chicks at 48 hrs, 6 and 8-d post-infection and 3, 10 d post-hatch to obtain total RNA for host cytokine and chemokine detection.

4.3.3 Bacterial isolation and identification

Bacterial swabs were plated on 5% Columbia sheep BA, m-Enterococcus and MacConkey agar and incubated at 37 C for 24-48 hrs. Semi quantitative analysis on plates were conducted in a scale from 0 to 4+ (153). To study bacterial growth in enriched cultures, bacterial swabs were inoculated in 3 mL of Todd Hewitt broth and incubated overnight at 37 °C on a shaking incubator to determine the number of embryos positive for bacteria. Bacterial isolates were identified using MALDI-TOF MS as previously described (372). To compare input and output pools of bacterial isolates from experiments, AMR profiles for 11 antimicrobials (*i.e.* AMP, CHO, ENR, ERY, FLO GEN, NEO, PEN, SPE, SXT and TYL) were conducted using Kirby Bauer disk diffusion method according to the CLSI guidelines (73). Antibiograms of input and output pools of bacterial isolates were compared at each time point and per organ to confirm identity of bacteria recovered from embryos following infection.

4.3.4 Histopathological examination

Histopathology of the YS, liver and lungs was conducted from three live embryos per group at 6 and 8-d post-infection from experiment A. YS, liver, lung and heart from any dead chicks following hatch were collected from experiment B. Tissue sections were preserved in 10% neutral

buffered formalin, embedded in paraffin, sectioned in 5 μm, and stained with hematoxylin and eosin (H&E) and Gram stain.

4.3.5 Scanning electron microscopy and transmission electron microscopy

Electron microscopy of YS of embryos from all the groups was performed at day-6 following *E. faecalis* infection (Experiment A). Tissue sections for Transmission Electron Microscopy (TEM) were osmicated with 1% osmium tetroxide then dehydrated through a graded ethanol series to propylene oxide and subsequently infiltrated with epoxy resin by gradual exchange. Blocked specimen were polymerized at 60 C overnight. Blocks were sectioned to 90 nm on a Leica Ultracut ultramicrotome and mounted on 200mesh copper grids. Images were collected using the Hitachi HT7700 TEM. Scanning Electron Microscopy (SEM) samples were paraffin embedded and affixed to glass slides by baking at 60 C. Samples were dewaxed in xylene and rinsed in 100% ethanol then sputter coated with 10 nm of gold. Images were collected using the Hitachi SU8010.

4.3.6 Molecular screening of putative virulence determinants of *E. faecalis*

Total genomic DNA from *E. faecalis* isolates were extracted using Qiagen DNeasy. Blood and Tissue kit according to manufacturer's instructions. Concentration of extracted DNA sample were determined by spectrophotometry at the wavelength of 260 nm and 280 nm. Virulence gene targets and PCR primers are listed in Table 4.1: Oligonucleotide primers used to amplify putative virulence determinants. Primers were designed as previously described (279, 396). All PCR reactions were carried out in a final volume of 50 μL reaction mixture containing 100 ng of DNA, containing 10X PCR buffer, 3.5 mM Magnesium chloride, 0.4 mM each of the four deoxyribonucleotide triphosphate (Fermentas, USA), 0.8 μM of each primer and 2 units of Taq DNA polymerase (Fermentas, USA). Samples were subjected to an initial cycle of denaturation (95 C for 5 min), annealing (at an appropriate temperature for 30 sec (Table 4.1), and elongation (72 C for 1 min), followed by 35 cycles in the thermocycler. Reference strain *E. faecalis* ATCC 29212 was used as a positive control. The amplification products were analysed by electrophoresis on a 1.0 % agarose gel at 100 V for 40 min in Tris-acetate-EDTA buffer and stained with ethidium bromide (20 μg/mL).

Table 4.1: Oligonucleotide primers used to amplify putative virulence determinants
agg; aggregation substance, *gelE*; gelatinase, *cylM*; cytolysins (*cylM*, *cylB*, *cylA*), *efaAfs*; adhesion-associated protein (Experiment A: *E. faecalis* associated pathology and cytokine response of chicken embryos)

Virulence gene		Primer(5-3)	Product size(bp)	Annealing temp(°C)
agg	R*agg*-F	AAGAAAAAGAAGTAGACCAAC	1553	55
	R*agg*-R	AAACGGCAAGACAAGTAAATA		
gelE	R*gelE*-F	ACCCCGTATCATTGGTTT	419	55
	R*gelE*-R	ACGCATTGCTTTTCCATC		
cylM	R*cylM*-F	CTGATGGAAAGAAGATAGTAT	742	53
	R*cylM*-R	TGAGTTGGTCTGATTACATTT		
cylB	R*cylB*-F	ATTCCTACCTATGTTCTGTTA	843	53
	R*cylB*-R	AATAAACTCTTCTTTTCCAAC		
cylA	R*cylA*-F	TGGATGATAGTGATAGGAAGT	517	53
	R*cylA*-R	TCTACAGTAAATCTTTCGTCA		
efaAfs	R*efaAfs*-F	GACAGACCCTCACGAATA	705	53
	R*efaAfs*-R	AGTTCATCATGCTGTAGTA		
cod	R*cob*-F	AACATTCAGCAAACAAAGC	1405	53
	R*cob*-R	TTGTCATAAAGAGTGGTCAT		
ccf	R*ccf*-F	GGGAATTGAGTAGTGAAGAAG	543	53
	R*ccf*-R	AGCCGCTAAAATCGGTAAAAT		

4.3.7 Reverse transcription quantitative PCR based determination of the putative virulence factor expression in *E. faecalis* during embryo infection

Samples of the intestine and liver were collected in 500 µL RNAlater from 3 live embryos per group at each time point in the experiment A. Samples were kept in -80 C until processing. Total RNA was extracted using Qiagen RNeasy Mini Kit according to manufacturer's instructions. Complementary DNA (cDNA) was prepared using QuantiTect Reverse Transcription kit according to manufacturer's instructions using random primers. Amplification, detection, and real-time analysis were performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, Calif.). SYBR Green I (Applied Biosystems) was used for the detection of the amplified product. Primers were selected for virulence gene expression level analysis as previously described (327). Amplification was carried out in a total volume of 20 µL containing

0.5× SYBR® Green master mix, and 2 µL of 1:5 diluted cDNA. The reactions were cycled 40 times under the following parameters: 95 C for 5 min initial cycle of denaturation followed by 95 C for 5 min denaturation, 60 C for 20 sec annealing, and 72 C for 30 sec extension steps. At the end of the PCR, the temperature was increased from 60 to 96 C at a rate of 0.5 C/min, and the fluorescence was measured every 5 sec to construct the melting curve. The housekeeping gene, 23S rRNA, and primers were used as previously reported (327). A non-template control was run with every assay, and all determinations were performed at least in duplicates to achieve reproducibility. Parallel to this experiment, 700 bp fragment of the *cpn60* gene from above prepared cDNA were amplified using conventional PCR described in section 4.3.6.

4.3.8 Determination of host cytokine gene expression in chicken embryos following *E. faecalis* and *E. coli* infection

Total RNA extraction and cDNA synthesis were conducted as described above. Host cytokine gene expression was determined in the intestine, liver or lung in experiment A and B using real-time PCR (Mx3000P qPCR system, Agilent Technologies) and TaqMan probes at 48 hrs, 6 and 8-d post-infection in embryos and 3 and 10 d following hatch in experiment C. The housekeeping gene used was 18S rRNA. The respective primer-probes and 18S rRNA amplifications in the same tube (20 µL total volume) were performed using Prime Time-Gene Expression Master Mix (IDT), 2 µL of cDNA template, and primers and probes as described in Table 4.2.

Table 4.2: Primers and probes for host cytokine gene expression detection by RT-qPCR (Experiment A: *E. faecalis* associated pathology and cytokine response of chicken embryos and Experiment C: *E. faecalis* together with *E. coli* associated cytokine response in embryos and neonatal chickens)

Primers/probe	Sequence
IL-1 Forward	5-GCTCTACATGTCGTGTGTGATGAG-3
IL-1 Reverse	5-TGTCGATGTCCCGCATGA-3
IL-1 Probe	5-(FAM)-CCACACTGCAGCTGGAGGAAGCC-(ZEN/IBFQ)-3
IL-8 Forward	5-GCCCTCCTCCTGGTTTCAG-3
IL-8 Reverse	5-TGGCACCGCAGCTCATT-3
IL-8 Probe	5-(FAM)-TCTTTACCAGCGTCCTACCTTGCGACA-(ZEN/IBFQ)-3
MIP-1 Forward	5-GGCAGACTACTACGAGACCAACAG-3
MIP-1 Reverse	5-ACGGCCCTTCCTGGTGAT-3
MIP-1 Probe	5-(FAM)-ACACAACACCAGCATGAGGGCACTG-(ZEN/IBFQ)-3
CXCR-4 Forward	5-TGCTGCCTCAATCCAATTCTT-3
CXCR-4 Reverse	5-CAAGGCATTTTGTGCTGATGTT-3
CXCR-4 Probe	5-(FAM)-ACGCCTTCCTGGGTGCCAAGTTC-(ZEN/IBFQ)-3
18S rRNA Forward	5-CGGCTACCACATCCAAGGAA-3
18S rRNA Reverse	5-GCTGGAATTACCGCGGCT-3
18S rRNA Probe	5-(HEX)-TGCTGGCACCAGACTTGCCCTC- (ZEN/IBFQ)-3

4.3.9 Statistical analysis

Fisher exact test was performed to determine the hatchability, mortality differences between groups. One way analysis of variance was conducted to determine the difference between cytokine and chemokine expression profiles among groups.

4.4 RESULTS

4.4.1 (A). *E. faecalis* associated pathology and cytokine response of chicken embryos

4.4.1.1 Hatchability and embryo mortality in chicken embryos infected with *E. faecalis*

Hatchability and cumulative embryo mortality was conducted between day 12 and day 21 of incubation (Figure 4. 1). No significant difference in hatchability or mortality was observed among groups of hatching eggs ($p>0.05$).

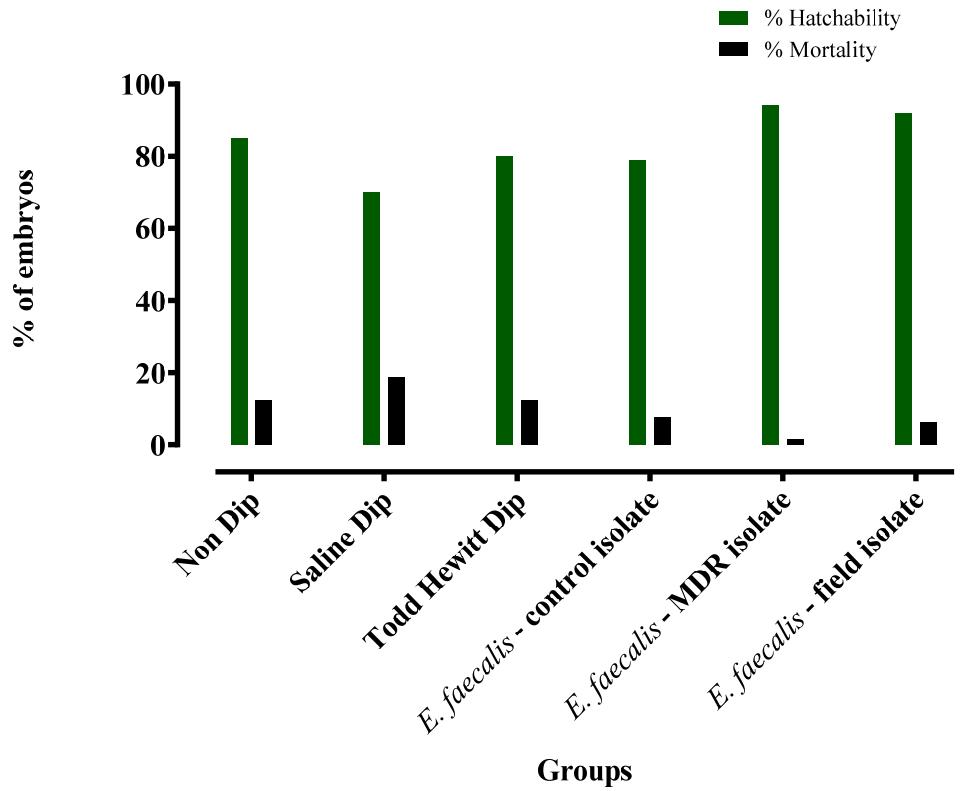


Figure 4.1: Hatchability and cumulative embryo mortality following *E. faecalis* infection. (Experiment A: *E. faecalis* associated pathology and cytokine response of chicken embryos.

4.4.1.2 *E. faecalis* eggshell penetration and colonization in internal organs of embryos

At 6-d post-infection, the MDR *E. faecalis* isolate was detected in the outer and inner compartments of eggs. The field strain and control strain were detected only in the inner compartments but not on eggshells. Colonization of the amnion by the MDR strain (60%) and the field strain (60%) compared to the control strain isolated from a healthy chicken (20%) was

different (Figure 4. 2). No *E. faecalis* was isolated from groups of eggs dipped in saline or Todd Hewitt.

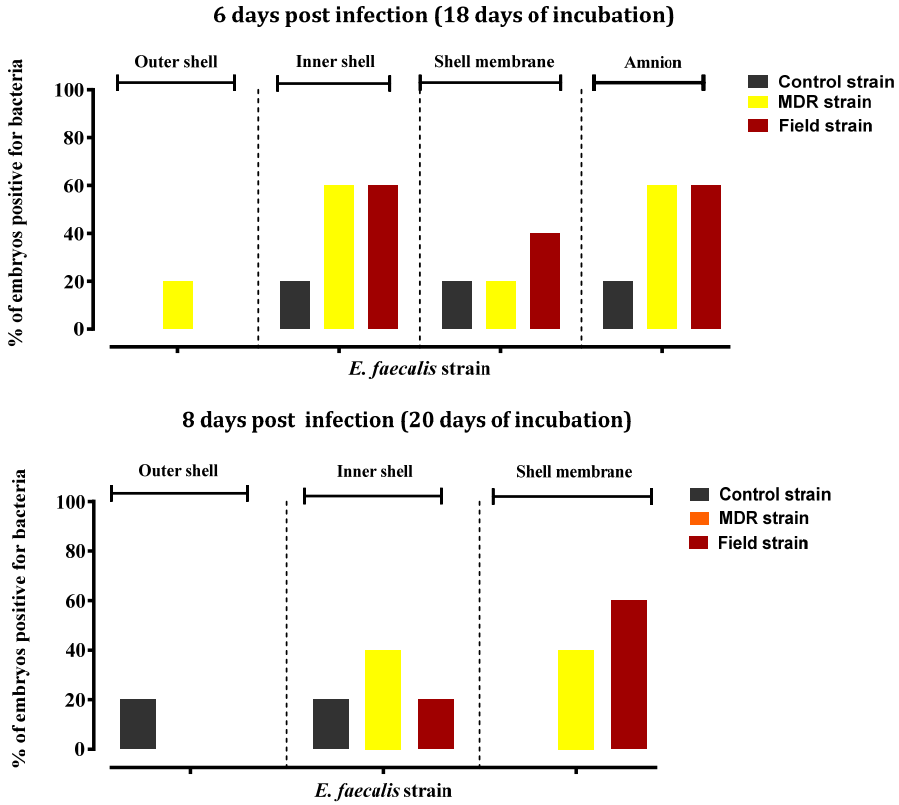


Figure 4.2: Isolation of *E. faecalis* from the surface outer and inner shell, and shell membrane at 6 and 8-d following exposure of incubating eggs to *E. faecalis*. (Amnion was not collected at day-20 of incubation since amniotic fluid was not sufficient for bacterial isolations.) (Experiment A: *E. faecalis* associated pathology and cytokine response of chicken embryos)

E. faecalis colonization in internal organs following egg infection was demonstrated in Figure 4. 3. *E. faecalis* load was enumerated following direct plate streaking. The MDR strain was isolated only from the liver (20%) and yolk (20%) but no other strains until 48 hrs post-infection. Colonization of *E. faecalis* in internal organs of chicken embryos increased with the time following egg infection. MDR *E. faecalis* was isolated 48 hrs following egg infection in the yolk and liver. At 6-d post-infection, *E. faecalis* load in the yolk, liver, intestine and lung reached 40%, 40%, 20% and 20%, respectively particularly with the MDR *E. faecalis* while yolk (20%) and brain (20%) were only positive for *E. faecalis* in the group infected with the *E. faecalis* strain isolated from a healthy chicken. The *E. faecalis* isolated from a field case of YSI colonized all internal organs at 8-d post-infection. *E. faecalis* isolate from a healthy chicken was only isolated at 6-d post-infection from yolk (20%) and brain (20%) where the minimal colonization rate observed compared to MDR *E. faecalis* or *E. faecalis* isolate from field case of YSI. AMR profiles were identical between *E. faecalis* isolated at the time of egg infection and from internal organs following embryo infections in respective groups.

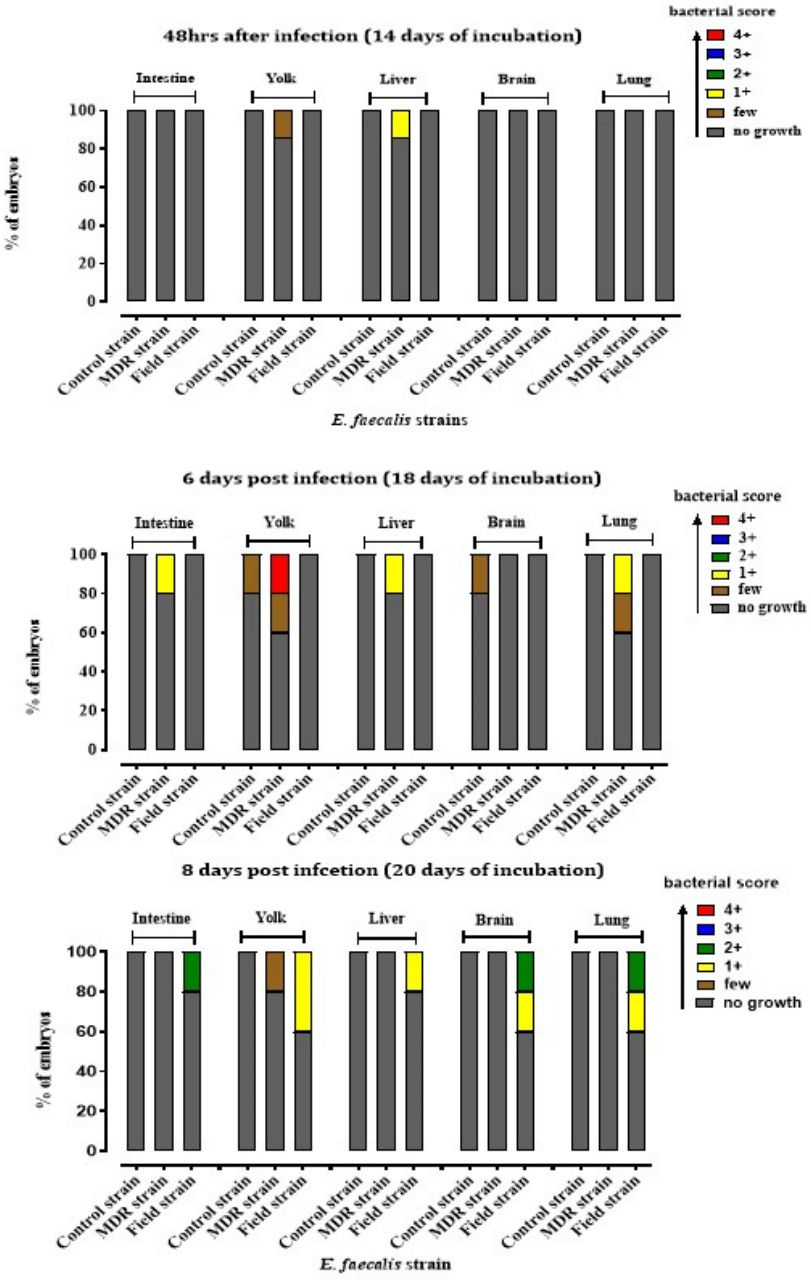


Figure 4.3: Isolation of *E. faecalis* from the yolk, intestine, liver, lung and brain following 48 hrs, 6-d and 8-d following exposure of incubating eggs to *E. faecalis*. (*E. faecalis* isolation was conducted in m-Enterococcus agar). Bacterial burden of systemic colonization with MDR *E. faecalis* was higher compare to other *E. faecalis* isolates. The field isolate of *E. faecalis* was detected predominantly at 8-dpost-infection from all organs including the brain. (Experiment A: *E. faecalis* associated pathology and cytokine response of chicken embryos).

4.4.1.3 Histopathology and electron microscopy

Histopathologic examination of tissue sections of the YS, lung and liver revealed colonization of Gram-positive cocci in the yolk, lung and liver (Figure 4. 4). There was no evidence of inflammation in any of the tissues in any of the groups exposed to *E. faecalis*. TEM and SEM demonstrated colonization of *E. faecalis* and biofilm like structures attached to epithelial cells of the YS 6-d following exposure of MDR *E. faecalis* (Figure 4. 5).

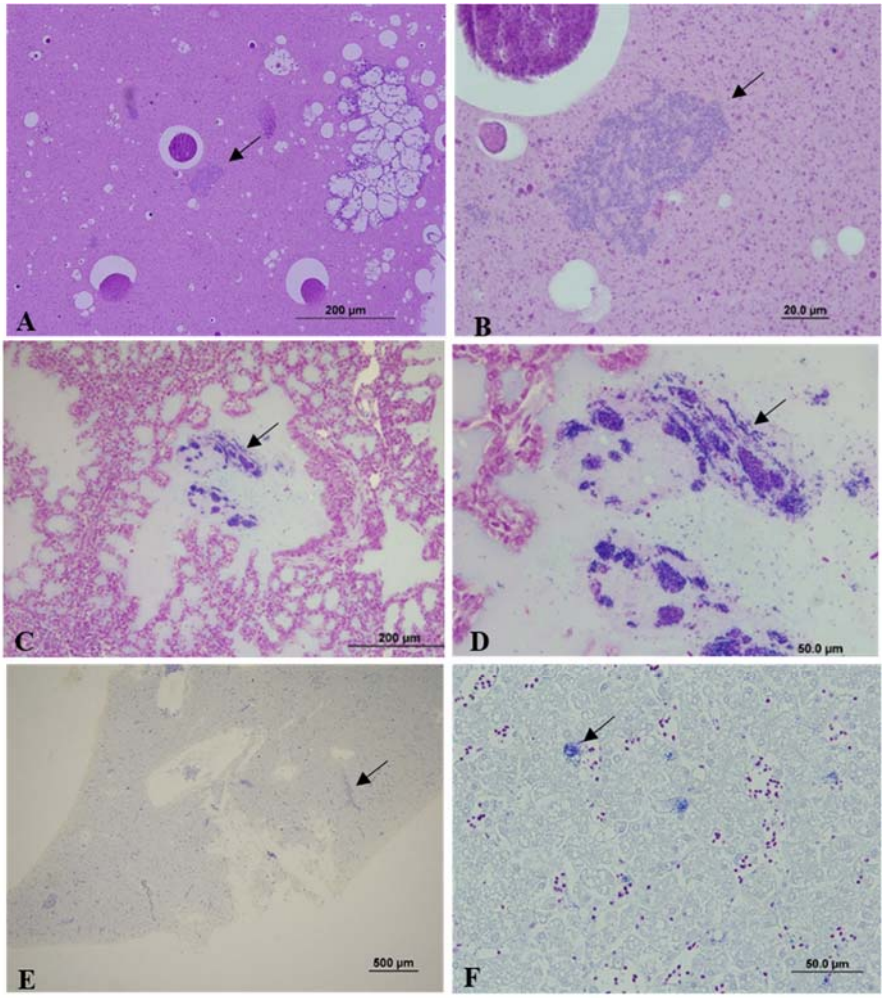


Figure 4.4: Gram and H&E stained sections of yolk, lung and liver 6-d following infection with MDR *E. faecalis*. Aggregates of Gram-positive cocci (black arrows) in the yolk, lung and liver. (A; x20 and B; x100= yolk; C; x20 and D; x60 = lung ; E; x20 and F; x60= liver) (Experiment A: *E. faecalis* associated pathology and cytokine response of chicken embryos)

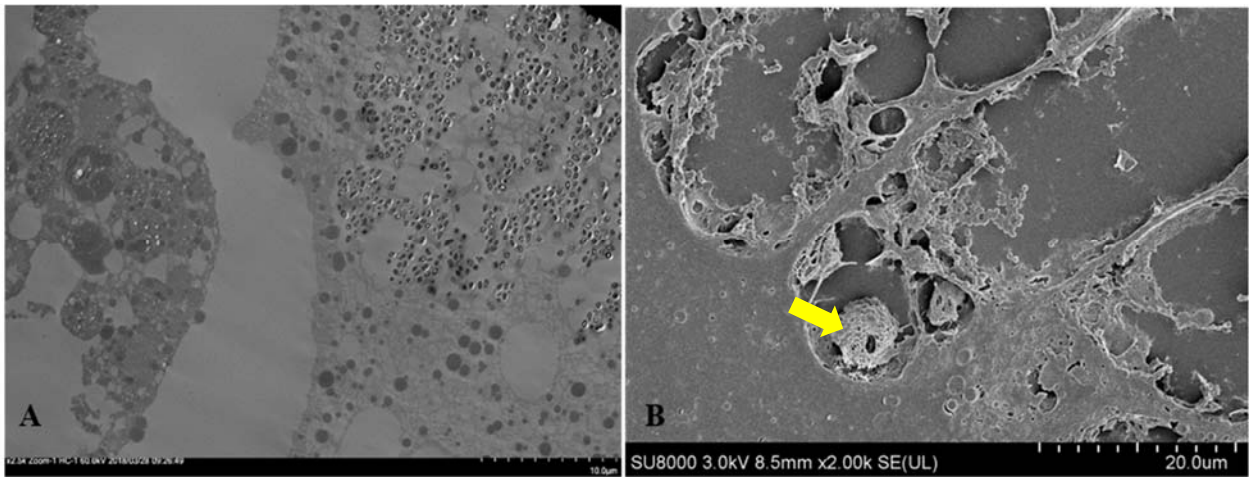


Figure 4.5: EM of MDR *E. faecalis* in the YS 6-d following exposure to *E. faecalis* TEM image (A) = (×2,000, ×8,000 magnification) illustrates heavy colonization of *E. faecalis* in the YS. SEM image (B) = (×2,000, ×8,000 magnification) illustrates biofilm like structure of *E. faecalis* attached to epithelial cells of YS (Yellow arrow). (Experiment A: *E. faecalis* associated pathology and cytokine response of chicken embryos)

4.4.1.4 Virulence gene expression of *E. faecalis* in chicken embryos during egg incubation

All the putative virulence genes were present in all three *E. faecalis* strains used in experiment A. The amplified genomic DNA products of putative virulence genes of *E. faecalis* were shown in Figure 4. 6. The presence of *E. faecalis* in the intestine, liver, YS and brain tissues of individual groups was confirmed by PCR amplification of *cpn60* (Figure 4. 7). The housekeeping gene was stable across all the time points and all the samples tested. However, virulence genes of *E. faecalis* were not expressed in any of the *E. faecalis* isolates recovered from any of the internal organs from any of the experimental groups during the entire length of the experiment.

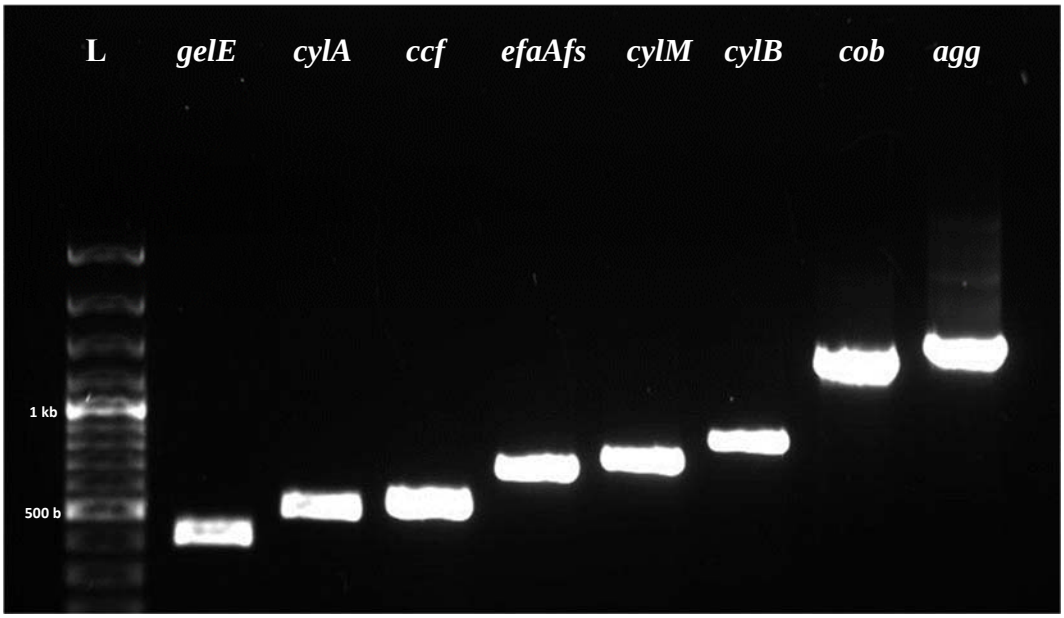


Figure 4.6: Figure 4. 1: Amplified genomic DNA products of putative virulence genes of *E. faecalis* (Experiment A: *E. faecalis* associated pathology and cytokine response of chicken embryos)

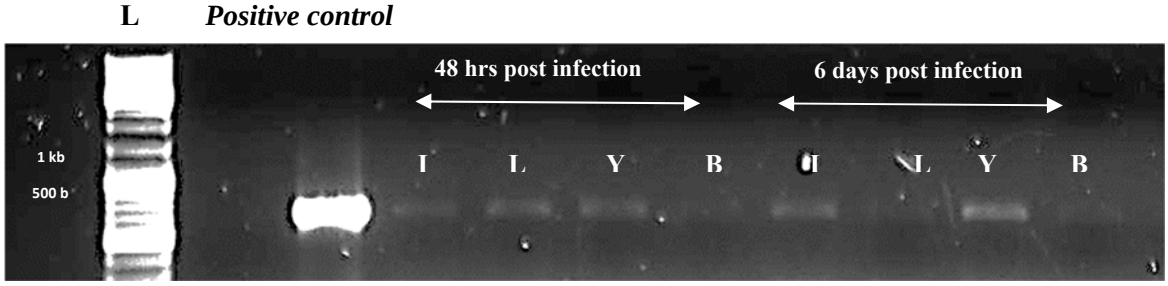


Figure 4.7: Amplified *cpn60* gene target from cDNA obtained from intestine (I), liver (L), yolk (Y) and brain (B) at 48 hrs and 6 days post infection. (Experiment A: *E. faecalis* associated pathology and cytokine response of chicken embryos)

4.4.1.5 Host cytokine and chemokine gene expression in chicken embryos following *E. faecalis* infection

The housekeeping gene, 18S rRNA was stable across all the time points and all samples tested. There were no significant differences among expression levels of cytokine and chemokine genes in any of the organs tested ($p>0.05$). Figure 4. 8 and Figure 4. 9 demonstrate expression of IL-1, IL-8, MIP1, and CXCR4 in the liver and intestine collected from groups of chicken embryos examined at 6-d post-infection of *E. faecalis*. Similarly, there were no significant differences

amongst expression levels of cytokine and chemokine genes among groups of chicken embryos exposed to *E. faecalis* or saline 48 hrs or 8-d post-*E. faecalis* infection (p>0.05).

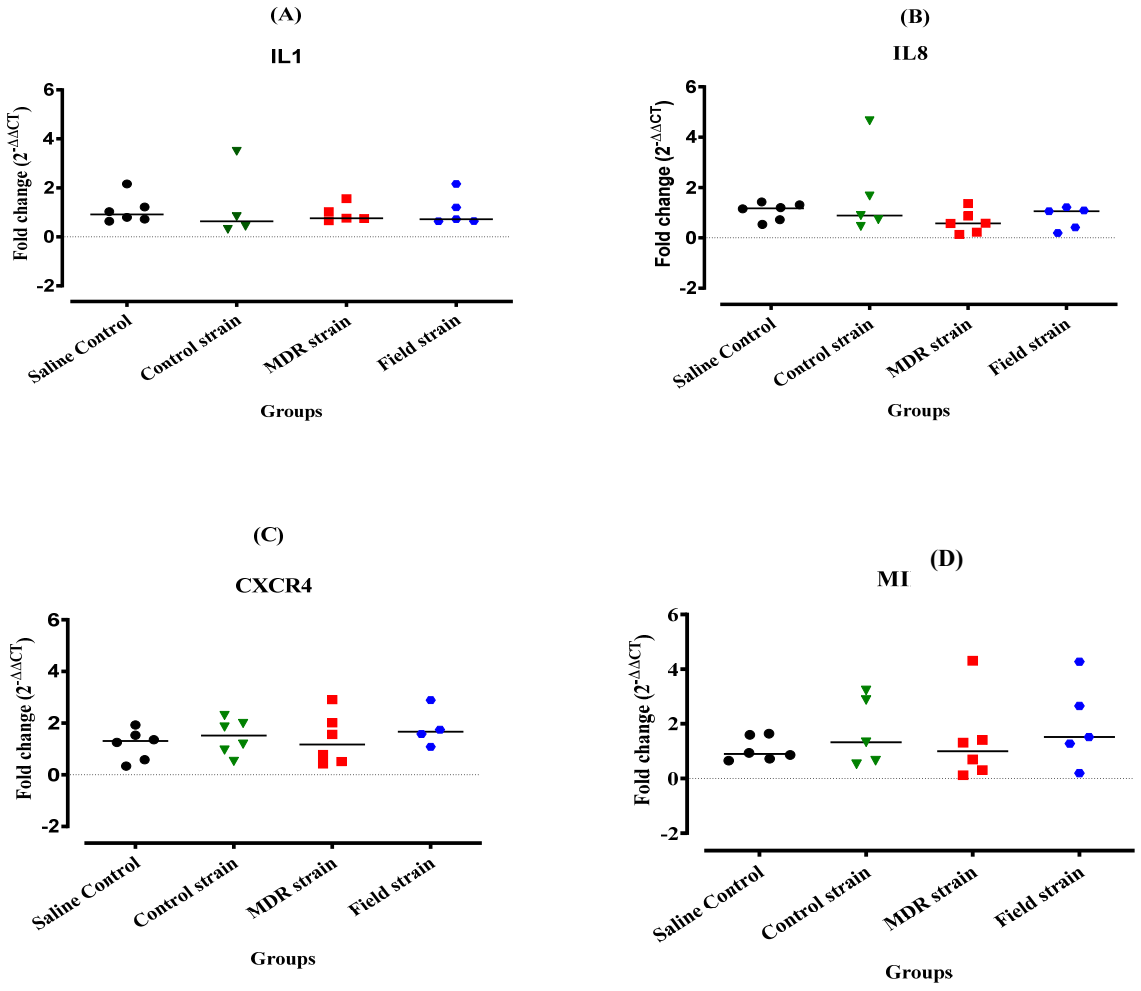


Figure 4.8: Cytokine and chemokine gene expression in the intestines at 6-d post-infection with *E. faecalis*. Each data point indicates an individual embryo. Horizontal bar represents the median. (Experiment A: *E. faecalis* associated pathology and cytokine response of chicken embryos)

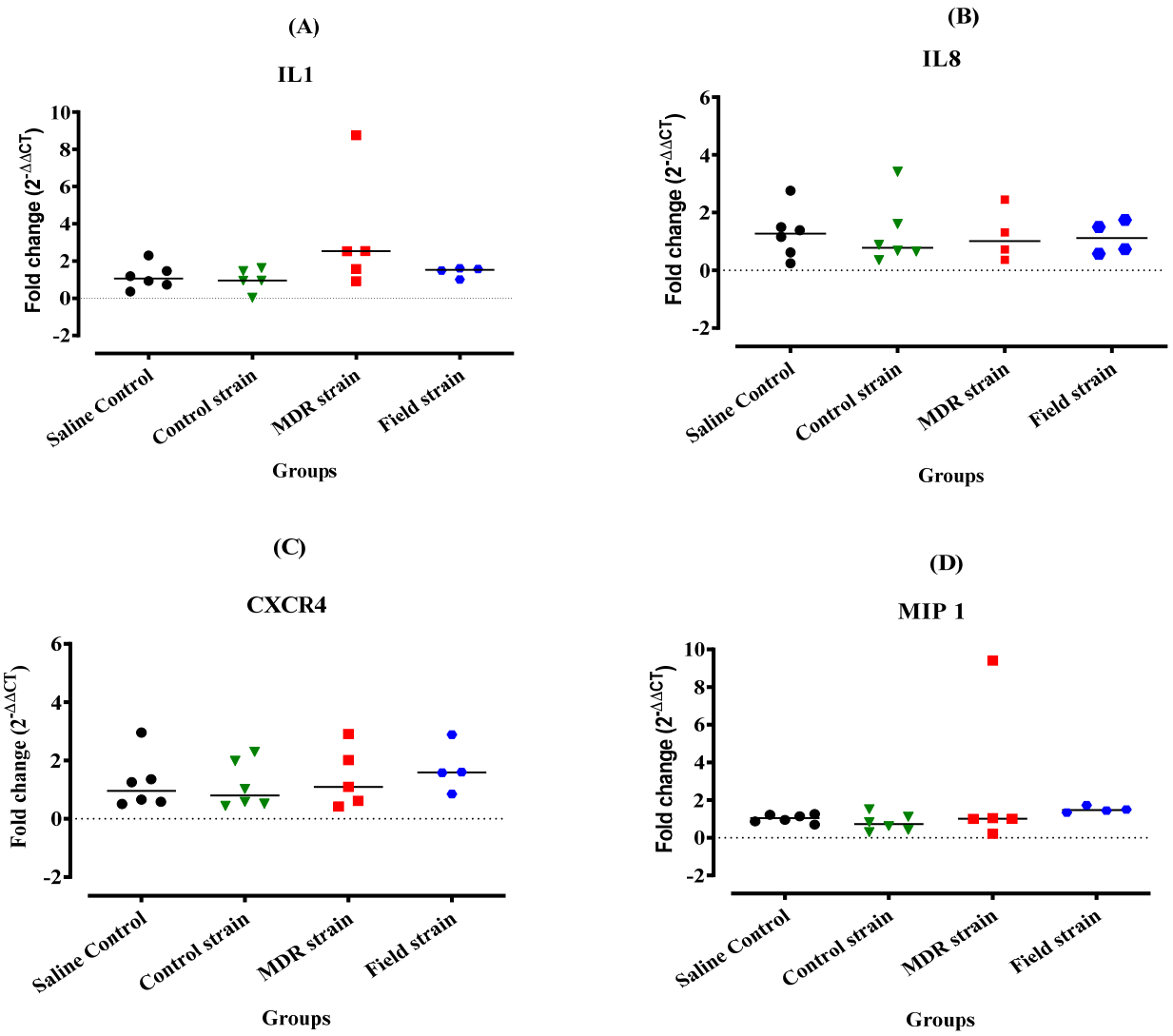


Figure 4.9: Cytokine and chemokine gene expression in the liver at 6-d post-infection with *E. faecalis*. Each data point indicates an individual embryo. Horizontal bar represents the median. (Experiment A: *E. faecalis* associated pathology and cytokine response of chicken embryos

4.4.2 (B). *E. faecalis* together with *E. coli* associated pathology in chicken embryos and neonatal chickens

4.4.2.1 Hatchability and embryo mortality in chicken embryos co-infected with *E. faecalis* and *E. coli*

There was no significant difference in hatchability or embryo mortality among groups of chicken embryos exposed to *E. faecalis* and *E. coli*, Todd Hewitt or the group exposed to no bacteria ($p>0.05$) (Figure 4. 10).

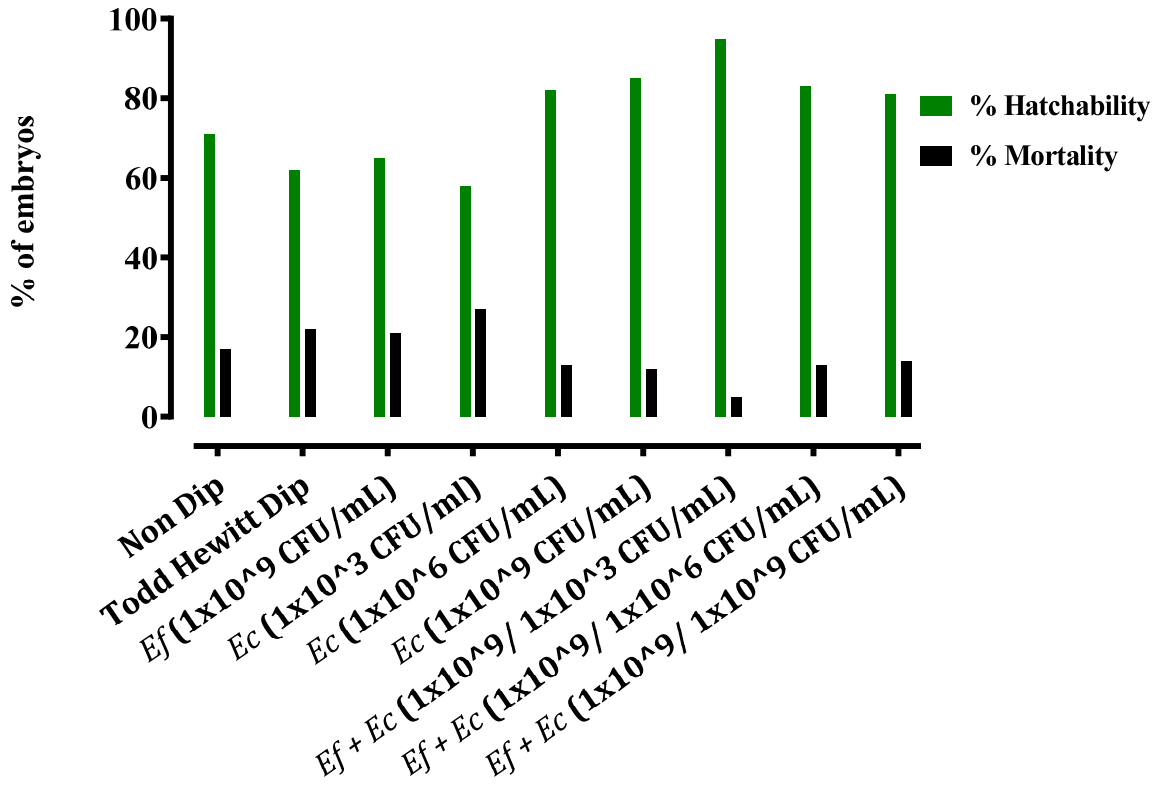


Figure 4.10: Hatchability and cumulative embryo mortality following chicken embryos exposed to *E. faecalis* alone, *E. coli* alone and co-infected with *E. coli* and *E. faecalis*. Ef; *E. faecalis*, Ec; *E. coli*. (Experiment B: *E. faecalis* together with *E. coli* associated pathology and in chicken embryos and neonatal chickens

4.4.2.2 E. faecalis or *E. coli* isolation from the yolk following chicken embryos exposed to *E. faecalis* and/or *E. coli*

Colonization of the YS with *E. faecalis* and *E. coli* at different time points during the incubation period is shown in Figure 4. 11. Bacterial counts were enumerated by direct plate streaking as mentioned above. At 48 hrs post-infection, *E. coli* was isolated from all groups except in the group exposed to $1x10^3$ CFU/mL *E. coli* or the group co-infected with $1x10^9$ *E. faecalis* and $1x10^3$ CFU/mL *E. coli*. The highest percentage of chicken embryos (40%) had isolation of *E. faecalis* from the YS at 48 hrs post-infection when chicken embryos co-infected with $1x10^6$ CFU/mL of *E. coli* and $1x10^9$ CFU/mL of *E. faecalis*. No bacteria was isolated from the groups not exposed to bacteria at any time point.

All the groups of chicken embryos exposed to bacteria were positive for bacterial isolation at 6-d post-infection in the group co-infected with *E. faecalis* and *E. coli* $1x10^9$ CFU/mL. The bacterial load was 100% at 6-d post-infection compared to 48 hrs post-infection (20%). Embryos infected with *E. coli* alone with $1x10^6$ CFU/mL dose had 100% bacterial colonization at 8-d post-infection where all embryos were positive for *E. coli* isolation. The number of embryos with bacterial isolation (enrichment culture) from the shell membrane, amnion and yolk are shown in Table 4.3. Bacterial isolation rates at different time points indicate that bacterial colonization is greater with the progression of incubation. Compared to *E. faecalis* alone infection, higher bacterial isolation was observed in the co-infected groups where isolations were prominent at 48hrs and 6-d post-infection.

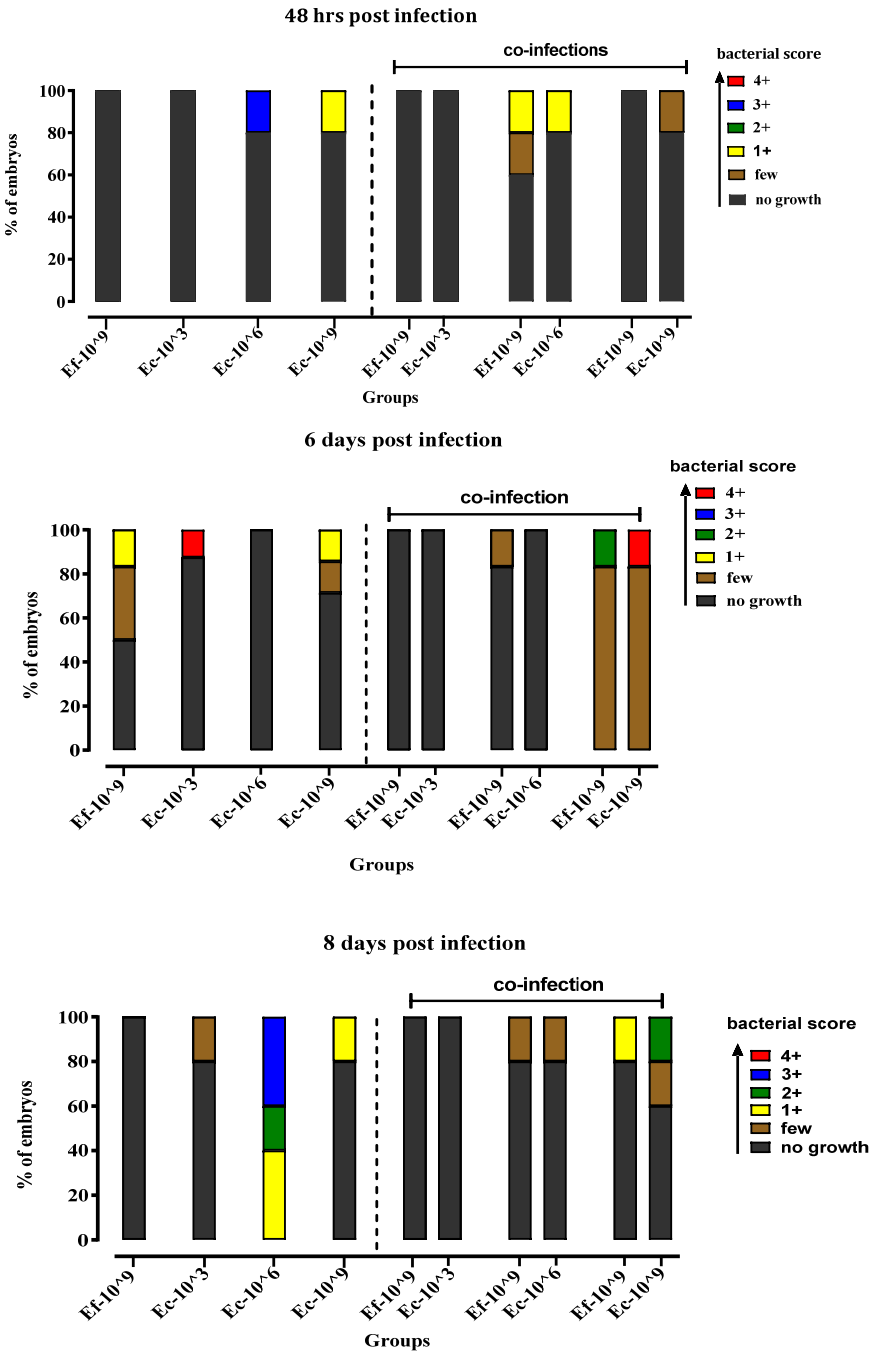


Figure 4.11: Isolation of *E. faecalis* (Ef) and *E. coli* (Ec) from the yolk at 48h, 6-d and 8-d following exposure of incubating eggs to different doses of *E. faecalis*, *E. coli* or co infected with *E. faecalis* and *E. coli*. Doses are indicated in CFU/mL as 1x 10^3- 1X 10^9. (Experiment B: *E. faecalis* together with *E. coli* associated pathology in chicken embryos and neonatal chicken

Table 4.3: Isolation of *E. faecalis* and/or *E. coli* from chicken embryos in enrichment culture. Enrichment cultures were obtained from shell membrane, amnion and YS in different time points following exposure to *E. faecalis* and/or *E. coli*. The empty boxes indicates no isolation was conducted. (Experiment B: *E. faecalis* together with *E. coli* associated pathology in chicken embryos and neonatal chickens)

Group- Time point	***Shell membrane***		***Amnion***		***Yolk***	
	E. faecalis	***E. coli***	***E. faecalis***	***E. coli***	***E. faecalis***	***E. coli***
*E. faecalis*10^9 CFU/mL- 48 hrs	0		0		0	
*E. faecalis*10^9 CFU/mL- 6d	28.6		28.6		57.2	
*E. faecalis*10^9 CFU/mL- 8d	20				80	
*E.coli*10^3 CFU/mL- 48 hrs		0		0		0
*E.coli*10^3 CFU/mL- 6d		12.5		12.5		12.5
*E.coli*10^3 CFU/mL- 8d		0				20
*E.coli*10^6 CFU/mL- 48 hrs		0		20		20
*E.coli*10^6 CFU/mL- 6d		50		33.33		33.33
*E.coli*10^6 CFU/mL- 8d		80				100
*E.coli*10^9 CFU/mL- 48 hrs		0		20		20
*E.coli*10^9 CFU/mL- 6d		42.9		28.6		71.5
*E.coli*10^9 CFU/mL- 8d		40				60
E. faecalis + *E.coli* (10^9/ 10^3)- 48 hrs	33.33	0	16.67	16.67	33.33	0
E. faecalis + *E.coli* (10^9/ 10^3)- 6d	60	0	80	0	60	0
E. faecalis + *E.coli* (10^9/ 10^3)- 8d	60	20			60	0
E. faecalis + *E.coli* (10^9/ 10^6)- 48hrs	20	0	20	20	20	20
E. faecalis + *E.coli* (10^9/ 10^6)- 6d	0	0	0	0	33.33	20
E. faecalis + *E.coli* (10^9/ 10^6)- 8d	0	20			60	80
E. faecalis + *E.coli* (10^9/ 10^9)- 48hrs	20	20	20	20	20	20
E. faecalis + *E.coli* (10^9/ 10^9)- 6d	16.7	33.33	16.7	50	83.33	50
E. faecalis + *E.coli* (10^9/ 10^9)- 8d	40	40			20	20

4.4.2.3 Neonatal chick mortality

The highest neonatal chick mortality of 13.33% was observed in the group of chicken embryos exposed to $1x10^{9}$ CFU/mL *E. faecalis* with $1x10^{6}$ CFU/mL *E. coli* (Figure 4. 12). The second highest neonatal chick mortality of 6.67% was observed in the group of chicken embryos exposed to $1x10^{9}$ CFU/mL *E. faecalis* with $1x10^{3}$ CFU/mL *E. coli*. Neonatal chick mortality of 6.67% was also observed in the group of chicken embryos exposed to $1x10^{9}$ CFU/mL *E. coli.* No clinical signs or mortality was observed in any other groups of chicken embryos exposed to *E. faecalis* or *E. coli*.

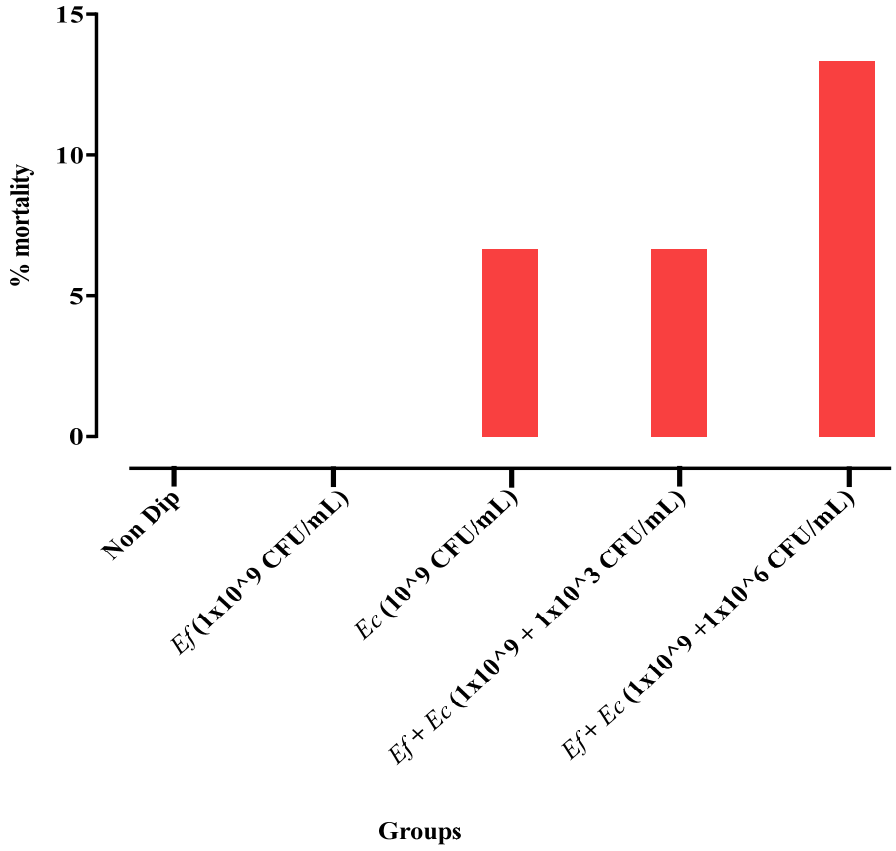
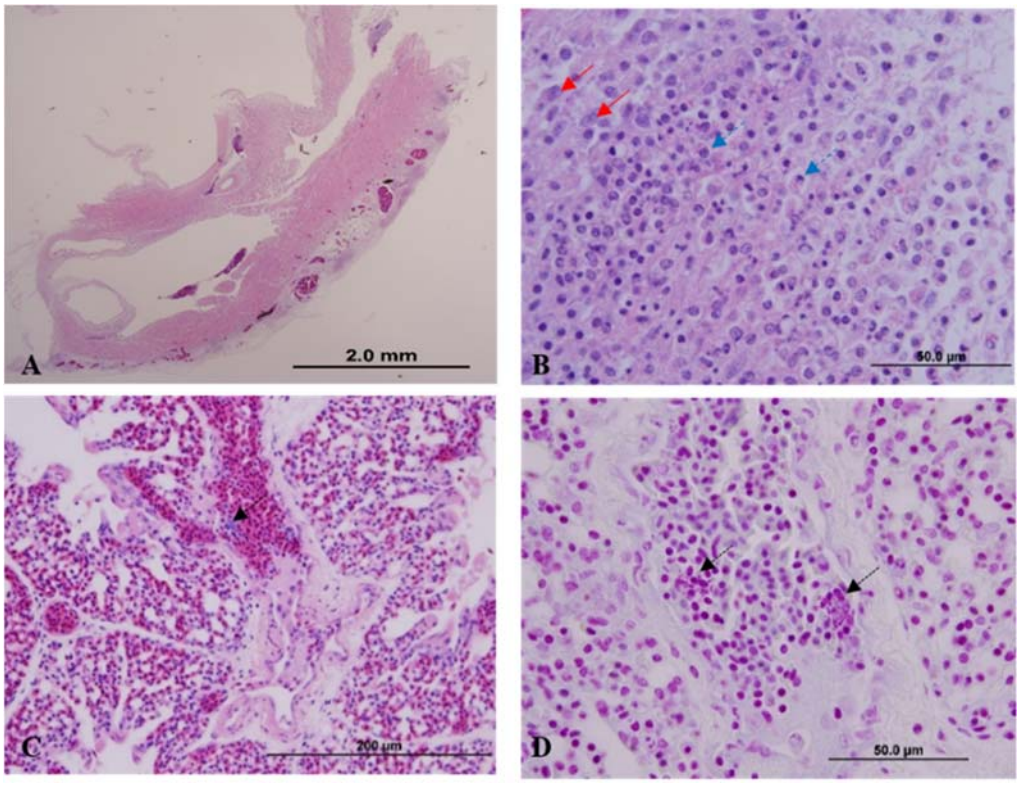


Figure 4.12: Cumulative neonatal chick mortality during day 0 – 7 post-hatch. **Ef; *E. faecalis*, Ec; *E. coli*** (Experiment B: *E. faecalis* together with *E. coli* associated pathology in chicken embryos and neonatal chickens)

4.4.2.4 Gross and histopathological lesions of neonatal chickens died following hatch

Macroscopic and microscopic examination of dead chicks revealed yolk sacculitis, pericarditis and perihepatitis (Figure 4. 13). Multifocal areas of necrosis were noted in the liver. The liver stained with Gram stain revealed Gram-negative rods representing *E. coli* in hepatic sinusoids (Figure 13; G and H). Epicarditis, pericarditis, myocarditis, along with infiltration of heterophils and macrophages were prominent in the heart. Gram-negative rods were prominent in the heart and blood vessels of lungs. YSM were thick and congested and multifocal areas had infiltration of heterophils and macrophages around necrotic debris. Gram-positive cocci and Gram-negative rods were present in the YS with necrotic areas. *E. faecalis* and *E. coli* were re-isolated in high numbers from infected tissues.


A
2.0 mm
B
50.0 µm
C
200 µm
D
50.0 µm

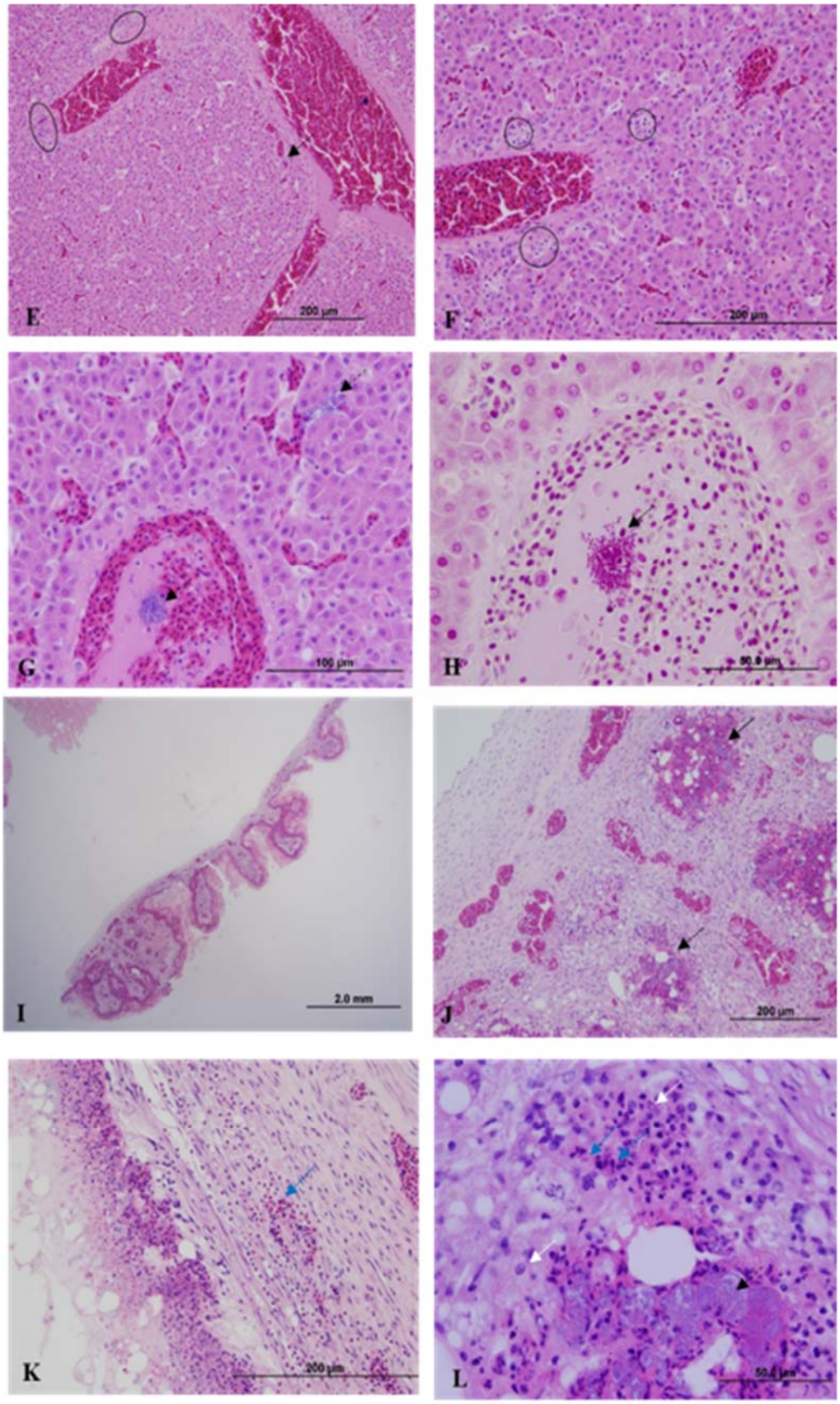

E
200 µm
F
200 µm
G
100 µm
H
50.0 µm
I
2.0 mm
J
200 µm
K
200 µm
L
50.0 µm

Figure 4.13: H&E stained tissue sections of the heart (A and B), lung (C and D), Liver (E, F, G and H) and YSM in dead birds (I,J,K andL). Heart had pericarditis (A; 2 X) with multifocal to coalesing areas with infiltration of heterophils (blue arrow) and macrophages (red arrow) (B; 100X). The lung contained rod shaped bacteria inside blood vessels (C; 40 X) and Gram-negative rods in the parenchyma (D; 100 X). The liver had multifocal necrotic areas (circled) adjcent to blood vessels (E; 20 X) with rod shaped bacteria (dotted arrow) in sinudoids and blood vessels (F; 40 X) (G; 60 X) (H; 100 X). YSM was diffusely thickened with inflammatory cells and fibrosis (I; 2 X) with bacterial aggregates (J; 10 X), heterophilic (K; 100X, L; 100X) and macrophage infiltrations in necrotic areas (L; 100X). Bacterial aggregates were identified in the YSM (L; 100 X). (Experiment B: *E. faecalis* together with *E. coli* associated pathology in chicken embryos and neonatal chickens)

4.4.3 (C). *E. faecalis* together with *E. coli* associated cytokine response in embryos and neonatal chickens

4.4.3.1 Neonatal chick mortality

The highest cumulative mortality of 27.5% was observed in the group co-infected with *E. faecalis* and *E. coli*. The cumulative mortality of groups infected with *E. coli* alone, *E. faecalis* alone and non-dipped were 17.5%, 2.5% and 5 % respectively. The highest mortality of 12.82% was observed at day-2 post-hatch in the group co-infected group with *E. faecalis* and *E. coli*. (Figure 4. 14). No macroscopic lesions were found in any of the dead birds observed until 9-d post-hatch. Mortality in groups infected *E. coli* alone or *E. faecalis* and *E. coli* on 10-d and 11-d had pericarditis, airsacculitis, perihepatitis, peritonitis or yolksacculitis.

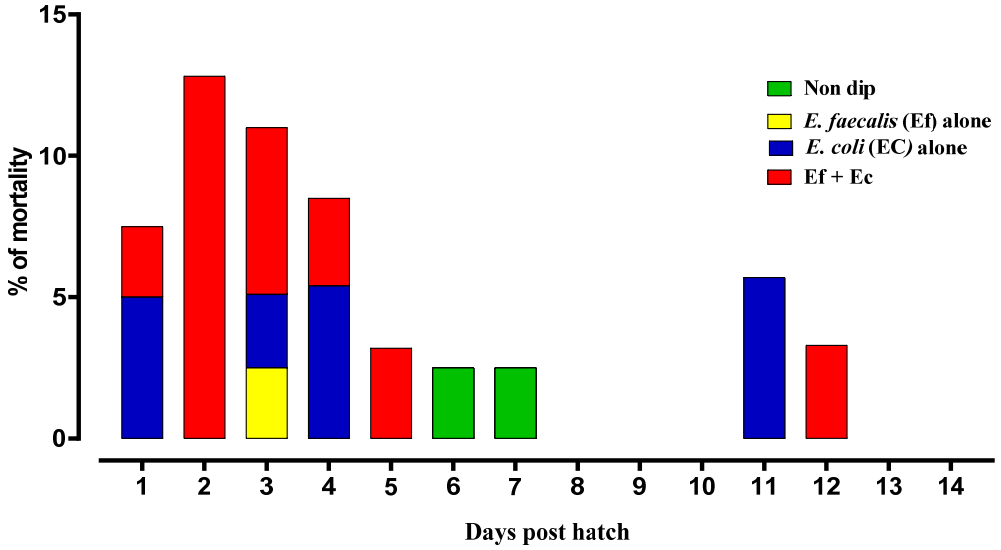


Figure 4.14: Neonatal chick mortality during 2 weeks post-hatch. Chicks from co-infected with *E. faecalis* together with *E. coli* had cumulative mortality of 27.5%. Of 27.5% cumulative mortality in the group co-infected with *E. faecalis* together with *E. coli* had a mortality of 12.82% on day-2 following hatch. (Experiment C. *E. faecalis* together with *E. coli* associated cytokine response in embryos and neonatal chickens)

4.4.3.2 Host cytokine and chemokine gene expression in chicken embryos and neonatal chicks following *E. faecalis* and/ *E. coli* infection

There was no statistical significance among groups for expression of IL-1, IL-8, MIP1, and CXCR4 in intestine or lungs at any time point ($p > 0.05$) but there was an upward trend in upregulation of IL-1 and CXCR4 at 8-dpost-infection (Figure 4. 15).

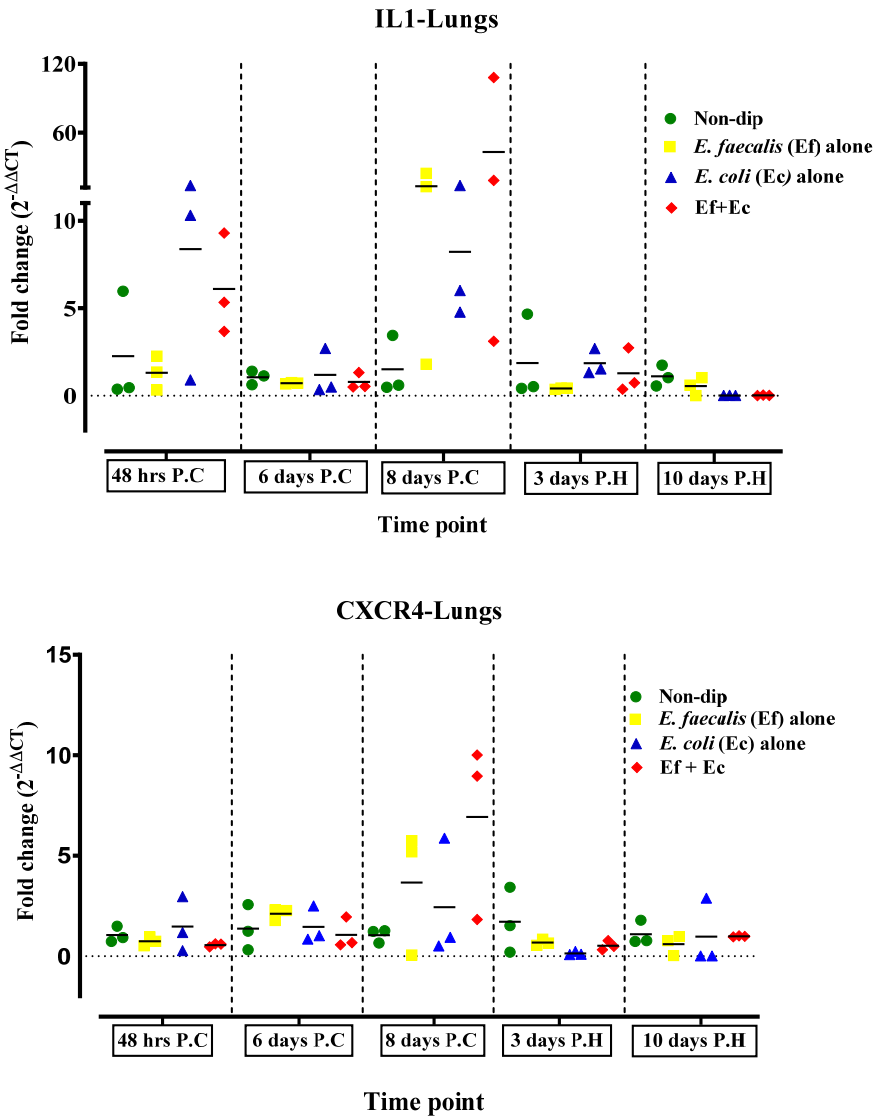


Figure 4.15: Cytokine gene expression in lungs at different time points following infection with *E. faecalis, E. coli* or co-infection of *E. faecalis* and *E. coli*. P.C; Post-challenge, P. H; Post-hatch. Each data point indicates an individual embryo/ chick. Horizontal bar represents the median. No significant difference among groups for IL-1 and CXCR4 expression in lungs ($p > 0.05$) but there was an upward trend in IL-1 and CXCR4 at 8-dpost-infection. (Experiment C: *E. faecalis* together with *E. coli* associated cytokine response in embryos and neonatal chickens).

4.5 DISCUSSION

E. coli and *Enterococcus* species associated infections and mortality in chicken embryos and neonatal chickens have been recently emerging worldwide (193). Contamination of fertile eggs of breeders with bacteria occur throughout the production cycle starting at broiler breeder farms, during egg storage and incubation in commercial hatcheries. Vertical transmission of certain bacterial species from the hen's reproductive tract to their progeny is hypothesized in several studies, particularly with APEC strains isolated from neonatal broilers with omphalitis and YSI (141). In a study demonstrating *E. faecalis* exposure via egg albumen led to arthritis in their progeny indicating possible vertical transmission (211). We have recently demonstrated an association of broiler chicken embryo mortality in commercial broiler chickens in western Canadian hatcheries due to coinfection of broiler chicken embryos with enterococci and *E. coli* (193). *E. faecalis* was isolated from the YS of the majority of dead embryos followed by *E. coli* in this study. Although it has been reported that chicken embryo mortality and YSI due to *E. faecalis* and *E. coli* were common in the commercial poultry industry, the pathogenesis of *E. faecalis* infections in chicken embryos and neonatal chickens is not clear. The objective of this study was to determine host pathogen interactions of *E. faecalis* and *E. coli* in chicken embryos and neonatal chickens.

We have conducted three experiments to demonstrate host pathogen interactions of *E. faecalis* and *E. coli* in chicken embryos and neonatal chickens: the first experiment was to demonstrate *E. faecalis* infection alone in chicken embryos and host response of embryos against *E. faecalis*, the second experiment to demonstrate the synergistic effects of *E. faecalis* and *E. coli* in embryos and neonatal chickens and the third experiment to demonstrate cytokine response of embryos and neonatal chickens. According to our knowledge, this is the first study that attempt to demonstrate the role of these organisms and the mechanisms in the pathogenesis of chicken embryos and neonatal chickens.

In the first experiment, we demonstrated that *E. faecalis* was able to transmit through the egg shell to shell membrane, amnion, liver, brain and intestines during the incubation period of without causing embryo mortality or negatively affect hatchability. The bacterial load of the MDR *E. faecalis* was higher compared to the field isolate of *E. faecalis* causing YSI or *E. faecalis* isolated from a healthy chicken at 48 hrs following exposure; *E. faecalis* load in embryos became similar among all isolates close to hatching. Although, the virulence factors associated with adhesion, colonization and cell damage were genotypically present in all *E. faecalis* isolates, no

expression of any of these genes occurred during embryo development. This may have been the reason for not having an inflammatory response against *E. faecalis* despite colonization in multiple organs during the incubation period of embryo development. It is also possible that *E. faecalis* down regulates immune responses of the host or escapes from the immune system of chicken embryos. This may have been the reason that there was no upregulation of host cytokine and chemokine genes until day-18 of incubation although bacterial colonization was observed in multiple organs during the incubation period of chicken embryos. It has been demonstrated in a mouse model of catheter-associated UTI, that *E. faecalis* was able to subvert macrophage activation by preventing NF-κB signaling pathway which controls the transcription of genes responsible for immune regulation and proinflammatory cytokines and chemokines that regulate recruitment and activation of immune cells (358). An experiment using a green fluorescent protein-expressing *E. faecalis* strain infected macrophage cell culture demonstrated that *E. faecalis* was able to survive in cytoplasmic vacuoles of macrophages without forming auto phagosomes, and was resistant to acidification after phagocytosis (404). Our results are in an agreement with findings of a study conducted to evaluate genomic differences among *E. faecalis* isolated from different habitats where they have found that the environment of origin had an influence on the shaping of the core genome *E. faecalis* for survival in different habitats (160). Furthermore, it has been demonstrated that antibiotic resistance modulates bacterial fitness and virulence potential, thus influencing the ability of pathogens to survive in a particular niche (139). We have found in our experiments that MDR *E. faecalis* had a higher colonization rate in organs compared to other *E. faecalis* strains. However, a commensal *E. faecalis* isolate that originated from the gut of an adult healthy chicken was able to penetrate, colonize and persist in the embryo, thus indicating the possibility of embryo infection following fecal contamination of eggshells.

The *E. faecalis* core genome contains about 60 putative virulence determinants associated with adherence, antiphagocytosis, biofilm formation, quorum sensing system, production of exoenzymes and toxins. Of these 60 genes we studied, only eight virulent determinants responsible for adherence, biofilm formation and cell damage were identified. We did not see any difference in the presence of these virulence factors among *E. faecalis* strains. Genomic analysis of different *E. faecalis* strains isolated from variety of environmental and clinical sources indicated that highest number of virulence factors were present in clinical isolates compared to environmental isolates

(160). Hence, it is important to compare core genome of isolates to understand the whole spectrum of virulence potential and study their pathogenicity.

A study conducted by Landman *et al.* has demonstrated that exposure of 9-day-old incubating chicken eggs to *E. faecalis* by dipping in a broth containing $1x10^8$ CFU/mL *E. faecalis* isolated from a case of amyloid arthropathy resulted in YS colonization of 90% of embryos at day 19 of incubation. Moreover, they reported a hatchability of 78% and mortality of 17% were in incubating eggs exposed to *E. faecalis* while the group not exposed to *E. faecalis* had 91% hatchability and 0% mortality (211). These observations were in an agreement with our results indicating that horizontal transmission and eggshell penetration of *E. faecalis* is common and a leading source of embryo infection. The same authors also demonstrated that direct inoculation of *E. faecalis* to YS or egg albumen at day-6 of incubation led to significantly higher embryo death within the 48hrs following infection. Moreover, egg inoculation at 9-d of incubation indicated that later infection of the YS may not have detrimental effects even though early embryonic stage inoculation or contamination does (211) (379).

Recently we demonstrated that 56% of dead chicken embryos had coinfection of *Enterococcus* species with *E. coli* and this polymicrobial infection associated synergism likely result death of chicken embryos (193). A similar observation has been made in a nematode model of *Enterococcus* infection and concluded that synergism of *Enterococcus* and pathogenic *E. coli* resulted in the death of nematodes by 4 d compared to deaths happening later in the groups infected with either *E. coli* or *E. faecalis* alone. (216). Although, there was no significant difference in chicken embryo mortality among groups of incubating eggs exposed to *E. faecalis, E. coli* or coinfection of *E. faecalis* and *E. coli*, neonatal chicken mortality was increased in groups co-infected with *E. faecalis* and *E. coli.* We have seen biofilm like structures produced by *E. faecalis* and this may be a mechanism by which *E. faecalis* evades from the immune system of chicken embryos. Increased mortality of neonatal chickens following coinfection with *E. faecalis* and *E. coli* may have been associated with developmental changes of the immune system of neonatal chicken in addition to synergism of *E. faecalis* and *E. coli*. We have demonstrated a pro-inflammatory cytokine IL-1 and chemokine receptor CXCR4 response against *E. faecalis* and *E. coli* close to hatch and this may reflect changes of the immune response of embryos during development. Moreover, this change of host response against *E. faecalis* and *E. coli* is likely responsible for high mortality of neonatal chickens in the 24 hours following hatch as shown in experiment C. The increased level of IL-1 in

the lungs, as we have seen in experiment C, may have an effect on alveolar function by triggering inflammation and consequently effecting chick survivability (347). It is documented that surface CXCR4 levels on neutrophils increase after extravasation into injured lungs due to endotoxins and promote chemotaxis (392). The neonatal chickens which died of *E. faecalis* and *E. coli* were septicemic and had macroscopic and microscopic lesions of septicemia and YSI. It has been reported that *E. faecalis* may promote *E. coli* biofilm formation in low-iron conditions by promoting siderophores formation and facilitating polymicrobial infections in a mouse model (167). It has also been reported that *E. faecalis* can subvert or evade immune-mediated clearance in a mouse model of catheter-associated UTI model, but the mechanisms were poorly understood. It is also possible that *E. faecalis* may be able to evade from the immune system of embryos since immune surveillance is likely different in embryos and neonatal chickens. It was demonstrated in a mouse model of catheter-associated UTI that *E. faecalis* can actively prevent NF-κB signaling pathway in macrophages in the presence of TLR agonists and during polymicrobial infection of *E. coli*. (358).

In summary, our findings suggests that *E. faecalis* can penetrate the egg shell, evade immune barriers in the egg and systemically colonize. Moreover, incubating eggs co- infected with *E. faecalis* and *E. coli* lead to synergism of *E. faecalis* and *E. coli* and resulted in increased mortality in neonatal chickens. In addition, the maturation of the immune system during embryo development may play a role against bacterial infections. Further studies are warranted to understand the cellular and molecular mechanisms associated with immune surveillance of neonatal chickens against bacterial pathogens.

Chapter 5 DISCUSSION AND CONCLUSIONS

Loss of embryos during incubation and neonatal broiler chicken mortality can cause a significant economic loss to the poultry industry (274, 307, 395). Chicken embryo death can be attributed to infectious or non-infectious agents (307). A number of bacterial species are responsible for embryo mortality; predominantly *Enterococcus* species and *E. coli* which have been isolated from non-viable chicken embryos (176, 275). Recently, an emergence of *Enterococcus* species associated YSI in neonatal broiler chicken was reported in many countries around the world, including Canada, but the reasons associated with the emergence of enterococcal infections in poultry are not understood (264).

Among diseases in the broiler chicken industry, YSI caused by bacteria in neonatal broiler chickens is a major problem (296). It is possible that majority of YSI initiate during the incubation of chicken embryos. Hatchery management practices including egg storage, incubation, and transfer to setters are critical points to monitor in order to achieve a good hatchability. Maintenance of proper temperature, humidity and good quality air are essential components of hatchery management (36). In addition to non-infectious causes good sanitary conditions and biosecurity are important to reduce losses during incubation and to improve hatchability. *E. coli* has been commonly isolated from non-viable broiler chicken embryos in commercial hatcheries around the world. Recently we have reported an enterococci associated chicken embryo mortality particularly with *E. faecalis* as the major cause compared to *E. coli* associated embryo losses in western Canadian hatcheries. The reason for emergence of *Enterococcus* species associated embryo mortality is not clear. Hence, one of the objectives of this study was to determine pathogenesis of *E. faecalis*-associated chicken embryo mortality and subsequent neonatal deaths due to YSI.

Chicken embryo death could be a result of many reasons, but as described in chapter one, 65.82% of yolk material from non-viable chicken embryos were positive for at least one species of bacterium. It was concluded that majority of broiler chicken embryo losses were due to infectious aetiologies. Fifty-six percent of non-viable chicken embryos had isolation of *Enterococcus* species and *E. coli* hence, we postulated possibility of a synergism of enterococci and *E. coli*. Among *Enterococcus* species isolated from non-viable chicken embryos, *E. faecalis* was the predominant species followed by *E. faecium. E. coli* and enterococci are commensal organisms of the GI tract of broiler breeder parents hence; it is possible that chicken embryos losses were due to contamination of hatching eggs during egg lay, incubation or storage of eggs.

It is not clear whether commensal *Enterococcus* species in GI tract of chickens were opportunistic pathogens or whether they become pathogenic when they synergize with other bacterial species particularly with *E. coli*. *Enterococcus* species and *E. coli* are equipped with multiple virulence factors; hence, synergism between enterococci and *E. coli* could change the pathogenesis of *Enterococcus* species-*E. coli* complex in chicken embryos. Overall, our study concluded that *Enterococcus*-associated embryo mortality was predominant in all three hatcheries investigated and suggested that MALDI-TOF MS technology can be applied for bacterial identification such as *Enterococcus* species, isolated from poultry. In our study, we were able to demonstrate that MALDI-TOF MS was able to identify *Enterococcus* species and *E. coli* up to species level with 97.18% and 100% accuracy respectively. Since concordance between MALDI-TOF MS identification and sequencing identification is 100%, MALDI-TOF MS can be used as a robust and effective tool in poultry diagnostics. We hypothesized that enterococci can synergize with *E. coli* and lead to an increased mortality of chicken embryos and neonatal chickens.

Enterococci and *E. coli* are equipped with multiple virulence factors hence the second objective our research program was to study AMR profiles of *E. coli* and *Enterococcus* species isolated from non-viable chicken embryos in western Canadian broiler hatcheries. The isolated bacteria were identified using MALDI-TOF MS and susceptibility testing was performed using the Kirby–Bauer disk diffusion method. *E. coli* isolates were resistant to TET, AMP, AMX, SSS, CEF, GEN and SPE at the rate of 54.4%, 50.9%, 42.1% 31.7%, 29.8%, 28.6%, 20.5% respectively. Among those, 34.1% of *E. coli* were MDR. The most common *E. coli* resistant phenotype was tetracycline resistance (15.3%). Our data showed a high degree of resistance of *E. coli* to β-lactam antimicrobials; AMP (42.1%) and AMX (50.9%). Although we have not studied *E. coli* in depth, it may be interesting to determine the spectrum of β-lactam resistance among *E. coli* isolates. Our data in regards to ampicillin resistance (50.88%) of *E. coli* is comparable with AMP resistance of *E. coli* isolated (43%) from poultry products in Canada by the CIPARS in 2016. A recent study has described the emergence of ESBLs encoding plasmids from *E. coli* isolates in poultry with a similar rate of prevalence as observed in humans which warrants regular monitoring of AMR in the broiler industry.

All of the enterococci isolates were resistant to at least one of the antimicrobials tested. The descending order of AMR of *E. faecalis* were; TET (73.1%), CEF (47.9%), BAC (43.9%), ERY (31.4%) and TYL (30.5%). MDR was detected in 35.9% of *E. faecalis* isolates, and 85.7%

of *E. faecium* isolates. The most common resistant phenotype of *E. faecalis* was TET + BAC while the most common phenotype of *E. faecium* was CEF+ NEO + TET + SXT + PEN. This study highlights the urgency of monitoring AMR patterns of *E. coli* and *Enterococcus* species in non-viable chicken embryos in poultry hatcheries as potential reservoirs of AMR. We have observed a significantly high incidence of MDR enterococci (44.9%) in this study. Although *E. faecium* lacks major virulence traits compared to *E. faecalis*, it had a higher degree of multidrug resistance than *E. faecalis*. An increased prevalence of MDR enterococci has resulted in a major challenge in therapeutic options in human medicine. Our results indicate the hazard of dissemination of these MDR traits to bacteria infecting humans via the food chain, or a spill-over in the environment; hence surveillance of MDR enterococci in the poultry industry may be recommended. Depending on the virulence of these MDR bacteria, embryonic mortality could occur, if not, survivors will harbour these MDR bacteria resulting in shedding of superbugs in the environment. Because of these reasons, continued monitoring of MDR bacteria may be necessary in the poultry industry.

Although, *E. faecalis* and *E. coli* infections of chicken embryos and neonatal chickens are common in the poultry industry the synergistic effects of *E. faecalis* with *E. coli* were poorly understood in chickens. Therefore, the third objective of this study was to investigate the host pathogen interactions of *E. faecalis* and *E. coli* in developing chicken embryos and neonatal period of chickens as discussed in the Chapter 3. Eggs at day-12 of incubation were exposed to *E. faecalis* and/or *E. coli* on the egg shell by dipping eggs for 30 sec in a solution containing different concentrations of *E. faecalis* and/or *E. coli* in order to study migration of *E. faecalis* and *E. coli* during egg incubation and following hatch in neonatal chickens. A MDR isolate of *E. faecalis* was able to colonize multiple internal organs of chicken embryos quickly, as compared to an *E. faecalis* isolate from a healthy chicken, without affecting viability or hatchability of embryos. Although, *E. faecalis* colonized internal organs of chicken embryos, no histopathological lesions of inflammation were observed in any of the organs. Moreover, no host cytokines/chemokines or virulent genes of *E. faecalis* were expressed during *E. faecalis* colonization in embryos. Although, no mortality of embryos was observed in groups of embryos exposed to both *E. faecalis* and *E. coli,* neonatal mortality reached 13.3% within seven days following hatch. These findings suggest that *E. faecalis* may down-regulate or evade host immune system of developing chicken embryos and vertically transfer *E. faecalis* to neonatal chickens. Furthermore, synergistic interactions of *E. faecalis* and *E. coli* in chicken embryos lead to an increased mortality in neonatal chicken. This

study highlights the complex nature and differences of interactions of *E. faecalis* in chicken embryos and neonatal chickens and resulting pathology and mortality of chickens.

In summary, our research findings emphasize the importance of proper management and sanitary conditions of hatching egg, hatchery management and storage conditions of hatching eggs. As we have demonstrated in our 3rd chapter, egg shell contamination during the production process is highly correlated with increased first week mortality in chicks; thus control measures need to be taken to minimize egg shell contamination. Since enterococci and *E.coli* are normal inhabitants of chicken gut and abundantly present in the environment, it is important to minimize bacterial contamination of hatching eggs. We have demonstrated the complexity of polymicrobial infections in chicken embryos and neonatal chicken. Thus good hygiene is essential to reduce bacterial contamination that usually leads to infection and subsequently minimize therapeutic use of antibiotics in the poultry industry. This practice can minimize selection of MDR bacteria in the poultry industry and further transmission to humans.

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